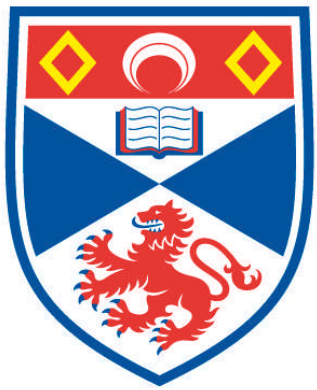
THE ROLE OF OXYTOCIN IN THE MATERNAL BEHAVIOUR OF THE GREY SEAL (HALICHOERUS GRYPUS)

Kelly J. Robinson

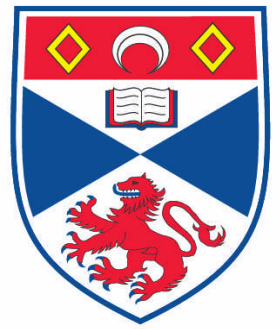
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



2014

The Role of Oxytocin in the Maternal Behaviour of the Grey Seal (*Halichoerus grypus*)

Kelly Robinson



This thesis is submitted in partial fulfilment for the degree of Doctor of Philosophy,
School of Biology at the University of St Andrews

January 2014

1. Candidate's declarations:

I, Kelly Robinson, hereby certify that this thesis, which is approximately 60,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in January 2010 and as a candidate for the degree of Doctor of Philosophy in January 2010; the higher study for which this is a record was carried out in the University of St Andrews between 2010 and 2014.

Date 21.01.2014 signature of candidate …….

2. Supervisor's declaration:

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 21.01.2014 signature of supervisors

3. Permission for electronic publication: (*to be signed by both candidate and supervisor*)

In submitting this thesis to the University of St Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and the abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker, that my thesis will be electronically accessible for personal or research use unless exempt by award of an embargo as requested below, and that the library has the right to migrate my thesis into new electronic forms as required to ensure continued access to the thesis. I have obtained any third-party copyright permissions that may be required in order to allow such access and migration, or have requested the appropriate embargo below.

The following is an agreed request by candidate and supervisor regarding the electronic publication of this thesis:

Access to printed copy and electronic publication of thesis through the University of St Andrews.

Date 21.01.2014 signature of candidate ……. signature of supervisors

Abstract

The neuropeptide hormone oxytocin plays an integral role in mammalian reproductive endocrinology and behaviour. It has been utilised to study the physiological factors driving maternal behaviour in both laboratory and domestic mammals, but few studies have successfully detected oxytocin in wild individuals, or linked detected concentrations to the behaviours they exhibit. Phocid seals present an excellent system in which to study oxytocin's effects on maternal behaviour in the wild. The energetic constraints placed on a phocid mother during the dependant period should cause strong selection pressure for behaviour that maximises reproductive success with the least cost to the mother. However in many phocid species, substantial variations in maternal behaviour persist. In order to investigate whether oxytocin plays a role in driving this variation, behavioural and hormonal datasets were collected from grey seal mothers and pups on two breeding colonies in Scotland.

A protocol for the detection of plasma oxytocin in phocid seals was successfully developed, along with the methodology to manipulate peripheral oxytocin concentrations to directly test the hormone's impact on behaviour. Both correlatory studies on natural oxytocin concentrations and behaviour in wild mother-pup pairs and manipulation experiments on newly weaned pups show that plasma oxytocin concentrations influence behaviours that makes mother – pup separation less likely. These include increasing the time spent in close proximity to each other, increasing the number of checks performed on the pup and reducing the aggressive behaviour directed towards the other individual. Additionally, plasma oxytocin could be used as an indicator of weak maternal bonds between mother and pup, which resulted in behaviours such as abandonment and fostering. This study highlights the potential of oxytocin for studying variations in behaviours critical to an individual's reproductive success and provides the methodological framework for studies on other wild species to be conducted in the future.

Acknowledgements

This work would not have been possible without the funding awarded to me by the National Environmental Research Council and SMRU Marine. Thank you to these institutions and to the International Society for Wildlife Endocrinology and the Society for Marine Mammology, which both provided me with travel grants so that I could present some of the findings of this research at their conferences in 2013.

To my supervisor, Dr Paddy Pomeroy, thank you for your support, encouragement and your unwavering belief in me throughout the duration of this endeavour. Thank you for giving me the opportunity to work alongside you, both at SMRU and in the field, and most of all thank you for introducing me to one of the most incredible places I have ever been, I can only hope I will get back to Rona one day. You have taught me so much, and I will be forever grateful.

To my co-supervisors, Dr Neil Hazon and Dr Sean Twiss, thank you so much for everything you have done for me. Neil, you welcomed me into your lab and always made time for me to ensure that I was on the right path. Your stories about your own lab experiences, your unfailing patience with me and your constant support through every stage of this PhD kept me coming into the lab, even when the machines stopped working, when it was gone midnight and when it seemed like extracting all those samples was impossible. Sean, you have now been teaching me for ten years, and you are, as you were when I first sat in your lectures for 'Introduction to Whole Organisms', an inspiration. Your enthusiasm, ingenuity and kindness are qualities I can only hope to emulate in the coming years as I pursue becoming a research scientist.

Throughout my time conducting this research there were many people who gave me their time, advice and assistance, and for this I am extremely grateful. I wish to particularly thank Dr Ailsa Hall, who taught me so much about ELISAs, what to do if they are being troublesome and collected plasma samples from harbour seals for this project, Dr Mike Lonergan, who gave me invaluable advice about R coding and statistics, Dr Lars Boehme and Dr Theoni Photopoulou, who collected plasma samples from Weddell seals for this project, Ryan Milne and Alicia Widmer, who gave so much time and effort to help me work with the seals at the SMRU captive facility, Ian

Johnston and Pete Baxter for all their support in the lab with logistics and equipment, Annika Samnee and William Robinson for entering my handwritten behavioural observations into excel, and last but not least, Simon Moss. Simon, I cannot even begin to list the things you have done for me and for this project over the years. You gave me so much time to discuss anything from the experiments detailed in this thesis to my concerns about the project or PhD experience in general, and your ability to make everyone laugh and to make anything possible in the field (including fantastic food!) continue to amaze me. My time spent on the Isle of May was some of the happiest months of my PhD and I hope to find an excuse to come out with you again soon.

To the people I was lucky enough to work with in the field, William Patterson, Ross Culloch, Hananh Wood, Matthew Bivins, Theoni Photopoulou, Paula Redman, Johanna Baily, Amanda Stansbury, Luke O'Connor, Vaida Natkeviciute and Martina Galicotto, thank you for those incredible seasons on those islands. Every season was different, fantastic and crazy, thank you for making them so memorable, for all the laughter and for never giving in when it came to weaner hauling!
To all my friends who have supported me during this project, you know who you are and how important you have been to me. Some of you have moved far away, some of you are still close to home, you are all stars and I could not have done this without you.

To my parents Malcolm and Alison, my sister Bethanne and brother Will, you have all had to live with my enthusiasm for the natural world, and I cannot thank you enough for a lifetime of encouragement and support in every conceivable way, from cheering me up when things looked bleak, to helping me pursue all my wildlife related hobbies, to actually assisting me in the field! Thank you so much, I would not be where I am today without you all.
Finally, to Rich. The love and support you have given me throughout this project is what has kept me going through the highs and the lows, and I can only hope that living with me for the last six months has not been too dreadful! You have done so much for me, from moving to Scotland to looking after me in these last weeks of writing, and I cannot even begin to thank you for it. Hopefully over the course of our lives together, I will be able to give you back at least some of what you have given me.

This thesis is dedicated to Tess, always loved, never forgotten.

Table of Contents

List of Figures

List of Tables

Chapter 1: General Introduction

All organisms must reproduce in order to successfully pass on their genetic material to future generations. There is huge variation in the physiological and behavioural reproductive strategies that have evolved across metazoans to carry out this crucial aspect of life history (Stearns and Hoekstra 2005, Extavour 2007). In the mammalian class, all species utilise sexual reproduction to produce their offspring (Rougier and Webb 2001, Stearns and Hoekstra 2005) and, uniquely in the animal kingdom, the female of the mated pair must provide for her infant(s) via the milk that only she can produce. As mammalian infants cannot survive the dependant period without their mothers, maternal desertion in mammals is rare compared to paternal desertion after fertilisation (Maynard Smith 1977). Therefore of the two sexes, mammalian females typically invest more of their energetic resources than males in each reproductive event due to the costs of gestation and lactation (Clutton-Brock and Harvey 1978). This selection pressure on the resources of the mother should therefore promote the prevalence of behaviours that maximise the likelihood of survival of the infant(s) while incurring the least energetic costs to the mother (Trillmich 2010).

Females of the *Pinnipedia* clade face additional energetic pressures while lactating compared to the majority of other mammalian species. As marine mammals they only feed at sea, however, they must give birth and nurse their offspring on land, where they cannot forage to replenish the energy they are expending (Cassini 1999). The three extant pinniped families each deal with this challenge differently. In contrast to all the other pinniped species, the one extant species of the Odobenidae family, the walrus (*Odobenus rosmarus*), is able to nurse while at sea and pups typically follow their mothers off the ice floes they were born on after only a few days (Miller and Boness 1983). Otariid seal and sea lion mothers leave their pups on terrestrial breeding colonies while they return to sea to forage, commuting back to the colony to feed the pup over several months or years in some species (Boness and Bowen 1996). Finally the majority of the phocid seals utilise a fasting strategy that requires them to remain on land with their pup for the entire dependant period, which is typically less than one month (Boness and Bowen 1996). The fasting strategy means that phocid mothers typically loose large proportions of their stored fat reserves and total body mass as their blubber layer is converted to milk (Costa *et al.* 1986, Bowen *et al.* 1987,

Iverson *et al.* 1993). With such high energetic costs to each yearly reproductive effort, phocid seals provide an excellent opportunity to study the physiology and expression of maternal behaviour under high selection pressure.

Grey seals (*Halichoerus grypus*) are phocids that breed annually on terrestrial or ice bound colonies (Hewer 1974). While on these colonies, females typically form a strong bond with their dependant pup so they can recognise it from unrelated ones (Burton *et al.* 1975). The formation of these bonds is crucial for the survival of the pup and the breakdown of mother-pup bonds is hypothesised to be the greatest ultimate cause of pup mortality on grey seal colonies (Baker and Baker 1988). It is thought that mothers also associate with specific adults during each breeding season to reduce aggressive interactions between individuals and the associated fitness costs (Pomeroy *et al.* 2005). However there are few studies investigating the basic ability to form social memories of unrelated conspecifics in any pinniped species. Maternal behaviour in grey seals also shows remarkable variety within and across individuals despite being constrained to an 18 day window (Fedak and Anderson 1982). There is significant variation in the quality of care a female gives (Fogden 1971), success rates of raising pups (Pomerory *et al.* 1999) and in the frequency of fostering behaviour exhibited (Perry *et al.* 1998). The physiological mechanisms underlying these behaviours have never been investigated from an endocrinological perspective in a pinniped species to date. Such an approach may provide significant insights into why potentially costly forms of maternal behaviours persist in a population.

Oxytocin, a neuropeptide hormone involved in female reproductive and social behaviour, is highly conserved across vertebrate organisms (Gimpl and Farhrenholz 2001). Oxytocin is well known for its roles in promoting parturition and lactation in mammals and additionally stimulates maternal (Lévy *et al.* 1995, Dwyer 2008) and pro-social behaviour (Ferguson *et al.* 2000, Kosfeld *et al.* 2005, Rimmele *et al.* 2009, Ross *et al.* 2009). Oxytocin has therefore been the subject of many studies looking to uncover the neurobiological and endocrinological mechanisms behind the variety of parental and social behaviours seen throughout the animal kingdom and the evolutionary forces driving their formation (for reviews see Uvnás-Moberg 1998, Ferguson *et al.* 2002, Broad *et al.* 2006, Heinrichs and Domes 2008). This thesis therefore looks to investigate the role the hormone oxytocin plays in the variation in maternal behaviour observed within and between individual grey seal mothers.

Additionally, the thesis will test the potential for grey seals to form enduring social bonds across breeding seasons.

1.1. The Role of Oxytocin in Maternal Behaviour

Oxytocin has long been known for its role in promoting birth (Dale 1906), its name meaning 'quick birth' in Greek (Gimpl and Farhrenholz 2001). It is used to induce labour in a number of species (Assali *et al.* 1958) including pinnipeds (Spotte and Stake 1982). After birth it then promotes formation of the bond between mothers and infants and the onset of maternal behaviour (Lévy *et al.* 1995). Oxytocin's fluctuations during parturition and the physiological changes they enact have been well studied in sheep (*Ovis aries*), which share several features of their maternal behaviour with grey seals including having few young per pregnancy, bonding and identifying offspring using scent and violently rejecting foreign offspring when approached by them (Kendrick *et al.* 1997). Oxytocin concentrations throughout the dependant period additionally have an impact on the behaviour a mother expresses, which in turn can have life long developmental consequences for the infant (Uvnás-Moberg 1998, Bales *et al.* 2007, Ross *et al.* 2009). Studies exploring oxytocin's role in the formation of mother-offspring bonds and as a moderator of maternal behaviour, therefore, may provide useful insights into the hormone's functions in phocid maternal behaviour.

The actual mechanism by which oxytocin induces birth is thought to be dependant on priming of the uterus by the changing ratio of oestrogen to progesterone during the late stages of pregnancy (Gimpl and Farhrenholz 2001). The rise of oestrogen in the uterus stimulates transcription and formation of oxytocin receptors in its tissue, thus making the uterus more responsive to circulating oxytocin (rats (*Rattus sp.)*, Bale and Dorsa 1997). The hormone can then stimulate contractions of the smooth muscle tissue required to expel the foetus (Nelson 2000). Vaginocerivcal stimulation caused by birth then stimulates the release of high levels of oxytocin in the mother, allowing the hormone to act on the brain regions responsible for forming the mother-offspring bond, and those needed to initiate maternal behaviour (Kendrick *et al.* 1991a). Evidence that oxytocin is vital for the formation of such bonds is provided in studies of sheep, where vaginocerivcal stimulation alone causes females to adopt foreign lambs as their own (Kendrick *et al.* 1991b).

The changes that occur in the brain after parturition from oxytocin action are substantial in sheep and rodents. The distribution of oxytocin receptors in the brain undergoes rapid change during birth and up to 24 hours afterwards (Insel and Shapiro 1992). Oxytocin acts on the medial preoptic area and mediobasal hypothalamus, which inhibit aggression towards infants and sexual receptivity respectively. The hormone also stimulates the paraventricular nucleus (PVN) and supraoptic nucleus (SON) to form a positive feedback loop to maintain high concentrations of oxytocin after parturition (Da Costa *et al.* 1996). In species which identify their offspring using olfaction such as sheep, another major site of oxytocin release at birth is the olfactory bulbs (Lévy *et al.* 1995). Its release causes changes in the cellular organisation of the structure, increasing the sensitivity of mitral cells to infant odors and allowing the brain to differentiate offspring odours with greater accuracy than normal. Imprinting on individual young therefore becomes possible in the time immediately after birth (Lim and Young 2006). Central oxytocin release additionally modulates the release of neurotransmitters crucial to olfactory memory such as γ-aminobutyric acid (GABA), to allow mother-offspring bonds to form (Lévy *et al.* 1995, Kendrick *et al.* 1992). In sheep this period of heightened sensitivity to offspring odours is only present approximately an hour after birth and if the mother-offspring bond has not formed by this time the female will not display maternal behaviour correctly (Alexander *et al.* 1986). Oxytocin therefore plays a crucial role in the formation of maternal bonds in mammals and additionally establishes positive feedback loops to perpetuate the maintenance of the bond (Nagasawa *et al.* 2012).

Certain maternal activities have been shown to incite both central and peripheral oxytocin release, most notably suckling, physical contact with and the sight, sound or scent of offspring in a range of mammalian species (humans (*Homo sapiens*): McNeilly *et al.* 1983, sheep: Dwyer 2008 and reviewed in Uvnás-Moberg 1998). Interaction between human parent-infant pairs has also been found to elevate peripheral oxytocin concentrations in both the adult and child (Feldman *et al.* 2010, Gordon *et al.* 2010, Weisman *et al.* 2012a) and mothers with secure, strong bonds with their babies have higher peripheral oxytocin concentrations than mothers with insecure, poor bonds (Levine *et al.* 2007, Strathearn *et al.* 2009). Oxytocin levels are thus kept high in parents when close to dependant offspring and interacting with them, activating several reward circuits in the brain so that maternal behaviour is then linked to a psychological reward (Da Costa *et al.* 1996). High oxytocin concentrations are

also correlated to optimal, as opposed to risky, maternal behaviour in humans (Atzil *et al.* 2011), to reduced aggression to offspring (sheep: Kendrick *et al.* 1997) and the amount a mother checks on her baby and maintaining close proximity between them (humans: Feldman *et al.* 2007). However, poor maternal care can arise when stimuli from the infant are not associated with oxytocin release and such rewards. In human mothers with insecure bonds with their babies, low amounts of plasma oxytocin are released when exposed to stimuli from their child compared to mothers with secure attachments to their babies. Their infant's presence additionally activates brain pathways associated with fear and disgust typically linked with rejecting an infant rather than nurturing it, while mothers with strong bonds activate reward pathways in the brain (Strathearn *et al.* 2009).

Some components of maternal behaviour have been experimentally shown to be triggered by elevated oxytocin concentrations in mothers via manipulation trials. Vasopressin, due to its structural similarities to oxytocin, has also been investigated but all studies so far have found it crucial for the onset of paternal, not maternal, behaviour (voles (*Microtus ochrogaster*), Wang *et al.* 1994, sheep, Da Costa *et al.* 1996, rats, voles and sheep, Kendrick 2000). Peripheral injections of oxytocin have been shown to decrease infanticide by female mice (*Mus musculus,* McCarthy 1990), increase maternal aggression towards foreign lambs in sheep (Kendrick 2000), increase pup retrieval in female voles (Bales *et al.* 2007) and increase the time spent with pups in meerkats (*Suricata suricatta*, Madden and Clutton-Brock 2011). Oxytocin Knockout (OTKO) mice have also been used to investigate maternal behaviour, and without a functioning oxytocin gene female mice display increased infanticide and aggression (Ragnauth *et al.* 2005) while OTKO pups are slower to seek out their mother upon separation and produce fewer ultrasonic calls to stimulate the mother to find them (Young *et al.* 1997). Mice with reduced oxytocin neurons in the PVN caused by *peg3* knockout also show deficits in nest building and pup retrieval (Li *et al.* 1999).

As well as regulating the formation of the mother-offspring bond and impacting on the quality and frequency of maternal care a mother expresses, neonatal exposure to oxytocin has life long developmental consequences for the infant (reviewed in Carter 2003). Vole pups exposed to peripheral oxytocin manipulations soon after birth showed differences in the receptor distributions for some peptides in central brain regions (Bales *et al.* 2007) and it also affected their reproductive physiology and

maternal and sexual behaviour as adults (Bales *et al.* 2004, Cushing *et al.* 2005). Primate infants deprived of their mothers have low cerebrospinal fluid (CSF) oxytocin concentrations and are unable to cope with stressful environments with oxytocin release like 'normally' raised individuals (Winslow *at al.* 2003). In the short term exposure to oxytocin as an infant additionally increases the body mass of rat pups and counteracts the effects on pups that maternal malnutrition causes during the dependant period (Sohlström *et al.* 2000, Olausson *et. al.* 2003).

Both naturally occurring elevations of oxytocin and experimentally heightened concentrations are documented to have an impact on the expression of maternal behaviour in a variety of mammalian species, making it an ideal candidate for investigating variation in the occurrence of these behaviours. As the expression of maternal behaviour frequently requires a large investment of a mother's resources to be successful, understanding the driving physiological factors behind its initiation and maintenance is a vital part of understanding variation in its expression, and the potential consequences any variation has for both mother and infant.

1.2. The Role of Oxytocin in Social Behaviour

Oxytocin has been linked to many aspects of initiating and maintaining social behaviour in mammals outside of the mother-offspring bond. It is able to initiate and promote social behaviour because it is released via a positive feedback mechanism in response to a number of stimuli that can occur in social contexts. Between unrelated bond partners, touch (voles, Cho *et al.* 1999, dogs (*Canis lupus familiaris)* and humans, Odendaal and Meintjes 2003 and chimpanzees (*Pan troglodytes)*, Crockford *et al.* 2013), close proximity to a bond partner (baboons (*Papio hamadryas ursinus*), Moscovice and Zielger 2012), the scent of and looking at familiar individuals (humans, Nagasawa *et al.* 2009) can all release oxytocin both peripherally and centrally in the brain. This release then acts on the brain in two main ways to encourage social behaviour. Firstly it inhibits the action of brain areas involved in fear such as the amygdala, reducing the perceived risks and stress of approaching another conspecific (humans, Petrovic *et al.* 2008). Secondly oxytocin activates the release of several neurotransmitters involved in reward or pleasure pathways in the brain, such as dopamine (voles, Gingrich *et al.* 2000). The neural pathways activated by oxytocin release have even been likened to those involved in drug addiction (Lim and Young

2006). Therefore, through its neurological effects in the brain, oxytocin encourages the approach of novel individuals and the formation of new social bonds, while providing a psychological reward for repeating interactions with familiar individuals.

There is much evidence supporting oxytocin's role in forming new social memories and bonds. It has been shown to be essential for the creation of new social memories in rodents, as OTKO mice, unable to produce the hormone, cannot 'remember' familiar individuals and treat them no different from novel ones (Ferguson *et al.* 2001). When given central injections of the hormone before being introduced to other individuals, OTKO mice show normal social recognition behaviour, and wild type mice given central injections of oxytocin antagonists show the same deficits in social memory as OTKO mice (Ferguson *et al.* 2000). Interestingly, OTKO mice showed no defects in non-social memory related tasks such as maze navigation and wild type mice were still able to recollect individuals they were exposed to before treatment with the antagonist. The OTKO experiments therefore indicate that oxytocin specifically affects the formation of new social memories in rodents. Due to its similarities in structure, vasopressin was also investigated in rodents with respect to social memory, but it has no effect on restoring social memory like oxytocin. Other studies indicate that vasopressin mainly acts on social behaviour in male animals, especially in the formation of monogamous pair bonds (Hammock and Young 2006).

Experiments manipulating levels of oxytocin in an individual's system have been extensively performed on humans to understand its role in social behaviour (Heinrichs and Domes 2008). Intranasal administration of the hormone has been shown to increase trust (Kosfeld *et al.* 2005, Baumgartner *et al.* 2008), empathy (Domes *et al.* 2007, Hurlemann *et al.* 2010), generosity (Zak *et al.* 2007), gaze to recognisable regions of the face (Guastella *et al.* 2007), the ability to recognise faces (Rimmele *et al.* 2009) and accelerates the formation of social memories (Savaskan *et al.* 2008). Oxytocin injections have even been used to treat autism, a condition in which poor social recognition and communication skills are common (Hollander *et al.* 2007). In other mammalian species, oxytocin has been used to induce the formation of social memories (mice, Ferguson *et al.* 2000), to extend the time individuals retain these memories (rats, Dluzen *et al.* 1998), to facilitate the formation of social bonds while reducing the frequency of investigative behaviour between two novel individuals (rats, Popik *et al.* 1992), to decrease aggression towards other conspecifics (meerkats, Madden and Clutton-Brock 2011), to increase the time spent in contact with a novel

partner (marmosets (*Callithrix penicillata*), Smith *et al.* 2010) and to increase the number of positive social behaviours individuals exhibit (voles, Carter 1998).

There is extensive evidence supporting oxytocin's role as a hormone promoting social affiliations, memories and recognition in mammalian species. While many studies use direct manipulations to determine oxytocin's effects on social behaviours and bonding, there are studies that document naturally occurring peaks in peripheral substrates to indicate positive social bonds between individuals. Both methodologies can be utilised to detect an individual's ability to recognise other conspecifics and adjust the behaviour they direct to one another as an indicator of simple sociality.

1.3. Maternal Behaviour in the Grey Seal

The maternal care given to a pup on a grey seal colony is a major factor in the likelihood of its survival both to weaning (Anderson *et al.* 1979, Baker 1984 and Twiss *et al.* 2003) and in the first year of life (Hall *et al.* 2001). Yet females of this species show remarkable variation in the quality of maternal behaviour they express to both their own, and foreign pups (Fogden 1971). From studies of model species, it is apparent that oxytocin regulates such differences in maternal care (Strathearn *et al.* 2009) but the hormone has never been investigated in any pinniped species or any wild animal species to explore variation in maternal behaviour despite the potentially useful insights that could be obtained from such work.

Grey seals breed on terrestrial or ice colonies, with a female producing one pup a year (Bonner 1989). Females typically show site fidelity to the colony and location within a colony they have previously given birth on (Pomeroy *et al.* 2000). In the United Kingdom, grey seals breed on terrestrial colonies around the coast, with thousands of individuals gathering in one location to pup and mate before returning to sea for the majority of the rest of the year. Remote islands are utilised for colony sites along with isolated areas of coast on the mainland (Baker 1984, Anderson and Harwood 1985). Both adult females and males on a breeding colony fast for the duration of their stay, and use nearby bodies of water, such as rainwater pools or the sea, to assist in thermoregulation while on the colony (Redman *et al.* 2001, Twiss *et al.* 2002).

Birth is an extremely rapid process in the grey seal, taking only a few seconds (Burton *et al.* 1975). As soon as the pup is born the mother will typically turn and sniff it, familiarising herself with its scent and forming the bond between them that will allow

her to recognise it while on a colony. The dependant period for grey seals is short for a mammal, typically spanning 18 days (Pomeroy *et al.* 1999). Pups emit calls which stimulate surrounding females to nurse, but scent is used to determine a pup's identity before accepting or rejecting it (Fogden 1971, Burton *et al.* 1975). Only in one Nova Scotian colony have female grey seals been shown to identify their pups using acoustic recognition (McCulloch and Boness 2000), much like otariid fur seals and sea lions (Insley *et al.* 2003). On the Isle of May in Scotland, no such ability was shown to exist (McCulloch *et al.* 1999) and it appears that these individuals rely heavily on olfactory recognition to distinguish their own pup from unrelated ones. The existence of a finite 'imprinting period' as described in other olfactory based mother-offspring recognition systems (such as those documented in sheep) has not been investigated in grey seals, although it has been reported that females make the most contact with their pups in the first hour after birth (Burton *et al*. 1975). Better understanding of the bonding process in grey seals could help explain the phenomenon of inaccurate pup identification, leading to abandonment or fostering.

A pup's chances of survival on a colony are almost completely dependant on its mother's provision of milk and protection from other aggressive adults. The mortality rates for pups on terrestrial colonies range from 14% to 35% (Boyd *et al.* 1962, Boyd and Campbell 1971, Anderson *et al.* 1979, Baker 1984), and approximately half of these deaths are thought to be ultimately caused by disturbance or breakage of the mother-pup bond (Boyd and Campbell 1971, Anderson *et al.* 1979, Baker and Baker 1988, Twiss *et al.* 2003). Therefore a female must not abandon, or leave a pup for long periods of time if it is to thrive. Pup survival to weaning (Mellish *et al.* 1999) and in its first year of life (Hall *et al.* 2001 and 2002) is also positively correlated with its mass and females typically loose 40% of their initial body mass during the dependant period generating fat rich milk to sustain their pup (Iverson *et al.* 1993), with suckling bouts occurring approximately every three hours (Haller *et al.* 1996). However females cannot simply invest large portions of their resources in every pup they give birth to. Females typically do not feed while on a colony and have a limited amount of resources they can devote to producing milk. If the amount of energy a female expends during one season exceeds a certain threshold it can negatively affect her breeding success the following year (Pomeroy *et al.* 1999). Mothers must therefore 'choose' how much of her resources should be devoted to her current pup,

while her own survival and future reproductive events may suffer should she invest too much in it (Pomeroy *et al.* 1999).

With pup survival so clearly dependant on it maintaining contact with its mother, and so much at stake in ensuring a pup gains enough weight to survive once weaned at 18 days, it would seem logical for females to be able to recognise and only feed their own pup. However despite this, fostering behaviour is not uncommon on grey seal colonies (Kovacs 1987, Perry *et al.* 1998). Fostering behaviour in grey seals is not thought to occur due to kin recognition as the females and pups involved are not related, giving no fitness benefit to the mother providing the resources (Perry *et al.* 1998). It is hypothesised that the cause of this behaviour is confusion in the mother over her own pup's identity, resulting in the bond between mother and pup weakening to the point of breaking (McCulloch *et al.* 1999). The time immediately after birth has been cited as the most crucial period for this bond to form, but females can spend significant amounts of time driving away gulls and perceived intruders at a pup's birth (Fogden 1971). If the pup's scent is not properly fixed into a female's memory as a result of such distractions, then confusion could easily arise when she is faced with many similar pups all calling to be fed, resulting in fostering behaviour.

Other opportunities for separation of mother/pup pairs and confusion over pup identity could arise when the mother leaves to lie in pools of water. The time a female spends away from her pup has been shown to be positively correlated with the distance her pup is from the nearest bathing pool, and as this distance increases so too does the probability of mother-pup pairs never being re-united (Redman *et al.* 2001, Twiss *et al.* 2000). The reasons behind why female grey seals do not have a more reliable method of identifying their own pup when their survival is so linked to the dyad remaining together remain unclear. Otariid species which commute from breeding colonies to feed at sea during lactation have very low levels of fostering behaviour, and have been shown to accurately recognise their pups from their calls (Insley *et al.* 2003), yet grey seal females in the UK seem to lack this ability (McCulloch *et al.* 1999). The motive behind a female trying to nurse up to four pups (Fogden 1971) when confused as to the identity of her own, considering her limited resources and the negative impact it could have on her future survival, also remains unclear.

As well as allowing investigation of the quality of bonds mothers and pups have, oxytocin concentrations provide an opportunity to investigate the variety in the quality

of maternal behaviour grey seals exhibit. On North Rona it has been deduced that over a 26 year period only 57% of the females on the colony produced 74% of the pups (Pomeroy *et al.* 1999). This variation in mother quality has been linked to a number of maternal variables, such as mass at parturition, date of parturition and location of pupping site (Pomeroy *et al.* 1999). Anecdotal accounts of behaviours ranging from extreme displays of 'devotion' to a pup (Boyd and Campbell 1971) to infanticide (K.J. Robinson, pers. obs) have also been observed in this species. However the factors linking maternal size and health, position on the colony and maternal success have not been explored from an endocrinological perspective. In humans high oxytocin concentrations soon after birth are associated with increasing the number of checks a mother makes on an infant and maintaining close proximity to the infant (Broad *et al.* 2006, Feldman *et al.* 2007), both behaviours seen in pinniped mothers which could be important for reducing the likelihood of separations occurring. The number of checks a grey seal mother performs on her pup has been proven to be consistent within individual mothers but varies greatly across individuals on a colony (Twiss *et al.* 2012a and 2012b). Oxytocin therefore seems a good candidate for investigating the variation in maternal care seen on grey seal colonies, and for investigating extreme forms of maternal behaviour such as fostering and abandonment.

1.4. Social Behaviour in the Grey Seal

All pinniped species must haul out on land or ice to give birth. This colonial breeding system can bring thousands of animals together at one time, creating the possibility for sociality. Grey seals are no exception to this rule, but are unique in the family for breeding on both ice and land according to what is locally available (Bonner 1989). Land colonies, such as North Rona and the Isle of May in Scotland, are structurally very different from ice based ones as they typically contain greater numbers of individuals in a smaller area. Selection pressure from male harassment, which is a major source of pup mortality in several pinniped species, is thought to be the largest factor driving increasing colony size in terrestrial environments across the pinniped clade (Camagna *et al.* 1992, Cassini 1999). However this trend does have its costs, and grey seal colonies have been shown to have increased female aggression towards other conspecifics and pups as densities of individuals increase (Redman 2002) and

increased competition for prime pupping sites within the colony (Twiss *et al.* 2000, Poland *et al.* 2008).

Two factors seem to govern the distribution of breeding female grey seals on terrestrial colonies, their proximity to the sea or freshwater pools (Pomeroy *et al.* 1994, Twiss *et al*. 2000, Redman *et al.* 2001) and the site the female previously gave birth (Pomeroy *et al.* 2000). Females will regularly pup near to access points to the sea (Fogden 1971) or pools of water if access to the sea is constrained on that particular colony, as is the case on North Rona (Anderson *et al.* 1975). The importance of having access to water is demonstrated in two patterns of female behaviour concerning their 'choice' of pupping site. The first females to arrive on terrestrial colonies will generally occupy the sites with the easiest access to water. Late comers must then occupy sites further away from water and are forced to commute from their pups to gain access to it (Twiss *et al.* 2000). Access to water therefore must be of great value to grey seal mothers. It is thought to aid thermoregulation during lactation (Twiss *et al*. 2002) and may also be used to regulate osmotic balance as females have been observed drinking from pools on colonies (Redman *et al.* 2001, Stewart *et al.* 2014).

Despite their dependence on access to water, females also show great site fidelity to a pupping site and will return close to the same spot every year they are on the colony (Pomeroy *et al.* 2000). Females also have been observed to have very little variation in the date they give birth every year (Pomeroy *et al.* 1994). If females are all returning to the same place to pup at a regular time, this raises the possibility that the same individuals are consistently adjacent to each other. It has therefore been proposed that such individuals are capable of recognising each other, and adjust their behaviour towards them (Pomeroy *et al.* 2005). Reduction of aggression towards familiar individuals is often cited as a crucial step towards sociality (Kleiman and Eisenberg 1973, Barrett *et al*. 2002, Estevez *et al.* 2003, Wolf and Trillmich 2007). Therefore the absence or reduction of aggressive behaviour between neighbours in grey seal colonies could provide useful insights into conditions that favour the evolution of simple sociality.

Reduction of aggression between neighbouring female seals would in theory be beneficial as it would allow them to invest resources expended during agonistic behaviour in their pup, increasing its likelihood of survival (Anderson and Harwood 1985). Several studies have attempted to detect and elucidate the presence of social

structure on grey seal colonies, with varied results. One study found evidence that throughout the terrestrial colony on North Rona there are areas of high and low social stability, and that in areas of high stability the levels of maternal efficiency were higher than elsewhere on the colony (Ruddell 2005). Another study found that in females not exhibiting site fidelity in pupping location, a higher number than expected choose to pup near a female they had previously associated with (Pomeroy *et al.* 2005). Both of these studies offer intriguing glimpses into the forces governing female distribution on one colony and its effects on maternal output. However, the recognition abilities of the grey seal have never been quantified between individuals outside of the mother-pup pair. Grey seals on a Nova Scotian colony also have been shown to be capable of identifying their pups acoustically, while Scottish seals on the Isle of May were not (McCulloch *et al.* 1999 and 2000). Therefore investigating the recognition capabilities that exist in this phocid species is a crucial first step in understanding any social structure that may be present within a grey seal colony.

One potential explanation for the presence of repeated associations on grey seal colonies and increased maternal efficiency in areas of social stability is the grouping of related animals together on a colony. However, studies analysing the genetic structure of North Rona have found little evidence to support this theory (Poland *et al.* 2008). Some kin clustering was found to be present on central, high quality areas of the North Rona colony (Pomeroy *et al.* 2001) but this does not explain the social structuring present on the remaining areas. Therefore while both female and male grey seals display site fidelity to their previous pupping site and breeding territory respectively (Twiss *et al.* 1994), and potentially show a degree of mate fidelity (Amos *et al.* 1995) and philopatry (Pomeroy *et al.* 2000) there must be another reason for grey seals to associate consistently with specific neighbours both within and across breeding season.

There is evidence pointing to seals remaining in the vicinity of, and hauling out on, their breeding colony throughout the rest of the year (Redman 2002, Pomeroy *et al.* 2005) and for individuals to form short term associations while at sea (Lidgard *et al.* 2012). Therefore there is potential for the creation and renewal of social associations when the animals are not breeding. However the behavioural mechanisms behind why more social females on a colony also perform better as mothers remain elusive, as areas of social stability have not been linked to reduced aggression as expected (Ruddell 2005). It has been hypothesised that if grey seals do form social

bonds that this occurs after weaning but before the pups leave the colony (Redman 2002, Pomeroy *et al.* 2005). Grey seal pups do not appear to be capable of social memory during the pre-weaning stage, and cannot recognise their own mothers on a colony (Insley *et al.* 2003). In rodent species the oxytocin receptor system in the brain has been shown to be developing during infancy but matches that of an adult by the age they are weaned (Wang and Young 1997). If this is the case in grey seal pups too, and the species is capable of forming social memories, then this would allow weaned animals to associate with animals that they leave the colony with. There is evidence from Satellite-GPS transmitter studies on grey seals that individuals at sea do not move independently from each other (Lidgard *et al.* 2012) and defining the recognition abilities for this species would be useful in interpreting such findings.

Whether grey seals can form social memories of unrelated conspecifics at all, how long they persist and at what stage in their life such memories are created remain unanswered questions. As oxytocin is crucial in the formation and re-enforcement of social memories and bonds, and the stimulation of social affiliation behaviour, it provides a unique opportunity to explore the species' recognition capabilities and the implications they have for the behaviour expressed towards other conspecifics on a colony.

1.5. Conclusions

Oxytocin has been implicated in regulating a range of bonding and maternal behaviours in mammals with both simple and complex social systems (Bielsky and Young 2004). Despite this, it has never been used to investigate the variation in social and maternal behaviour seen in any pinniped species or any wild mammal species, including the grey seal. There have been several studies investigating the colony dynamics of this species using a variety of behavioural and spatial modelling techniques, analysing the presence of stable inter-annual associations between mothers (Pomeroy *et al.* 2005) and generating indices of social stability within a breeding colony (Ruddell *et al.* 2007). While these studies indicate that a form of social structure may be present on grey seal colonies, no studies have been conducted on the grey seal's fundamental ability to form social bonds outside those existing between a mother and her pup. If grey seals are shown to be capable of social

recognition, then this will provide vital support for studies investigating the existence of geographically and temporally stable social bonds on breeding colonies.

Understanding oxytocin's role in maternal behaviour could greatly increase our knowledge of the forces governing a female seal's behaviour during the 18 days she has with her pup. Defining the physiological basis for initiating and regulating maternal care would potentially help clarify why some females exhibit fostering behaviour (McCulloch *et al.* 1999), some abandon their pups (Fogden 1971) and others continue to guard and nurture them even after death (K.J. Robinson, pers. obs), potentially at the cost of reduced reproductive success and survival the following year (Pomeroy *et al.* 1999). By measuring and manipulating oxytocin in grey seals, the physiological mechanisms governing maternal and social behaviour in this species can be defined. If the hormone does act in seals in a similar way to the primates, ungulates and rodents already investigated, then the existence of inter-annual associations between breeding females and the sometimes maladaptive maternal behaviour seen on grey seal colonies can be better understood.

1.6. Thesis Structure

The aim of this thesis is to explore the role of the hormone oxytocin in stimulating maternal and social behaviour in grey seals by correlating behavioural observations recorded from wild, breeding grey seals to the oxytocin concentrations present in their plasma. The effects of oxytocin on the behaviour of this species will then be directly tested using hormone manipulations in both mothers and in newly weaned pups that are about to leave the breeding colony. In order to successfully carry out this study, reliable, accurate protocols for the detection, quantification and manipulation of oxytocin concentrations in grey seals were developed.

The first objective of this thesis was to validate a protocol for collecting and analysing plasma and milk samples from phocid seals to measure the oxytocin concentration they contain (Chapter 3). In Chapter 3 several of the different collection and analytical protocols outlined in the existing literature are tested for their accuracy and consistency to determine the most suitable one for reliably detecting phocid oxytocin. Potential sources of variation such as restrain duration, freezer temperature for sample storage and the use of chemical restraint during sampling were also investigated.

The second objective was to describe any correlatory relationships that exist between the plasma oxytocin concentrations and the behaviour exhibited by wild mothers and their dependant pups on two grey seal breeding colonies in Scotland (Chapters 4 and 5). Detailed behavioural observations were taken from mother-pup pairs across three breeding seasons from 2009 – 2011 to provide an accurate record of the behaviour exhibited, and were combined into categories that accounted for the greatest amount of variance in the data (Chapter 3). These categories were then analysed with plasma oxytocin concentrations from the mothers and pups to detect any relationships between the two (Chapter 5).

The third objective was to directly manipulate plasma oxytocin concentrations in individuals to determine the direct effects the neuropeptide hormone has on behaviour (Chapter 6 and 7). Extensive development of a suitable dose and delivery method was performed to generate an oxytocin dose which would have the greatest potential to reach central brain regions and impact on behavioural expression despite being administered into peripheral circulation (Chapter 6). A series of manipulation experiments were then conducted using free ranging individuals and individuals in a semi-wild pen environment to directly test the effects of elevated oxytocin concentrations on the behaviour an individual expresses (Chapter 7).

The fourth objective was to test the recognition capabilities of grey seal conspecifics outside of the mother-pup dyad (Chapter 8). Individuals of similar ages were observed in a semi-wild pen environment with both novel conspecifics and those they had previously co-habited with. This would allow detection of any changes in behavioural frequencies across the two types of trial, to provide evidence for or against the grey seal possessing individual recognition abilities.

Finally the overarching results and implications of the thesis are discussed in Chapter 9, along with potential future directions for this work.

Chapter 2: General Methodology

2.1. Ethics Statement

This study obtained samples from free-ranging grey, harbour (*Phoca vitulina*) and Weddell seals (*Leptonychotes weddellii*) and from captive harbour and grey seals held under UK Home Office licence at the Sea Mammal Research Unit (SMRU) in St Andrews, Scotland. All capture and handling procedures were performed under Home Office project licence #60/4009 and conformed to the UK Animals (Scientific Procedures) Act, 1986. All research was approved ethically by the University of St Andrews Animal Welfare and Ethics Committee.

2.2. Study Sites

Field work was conducted on two grey seal breeding colonies in Scotland. Both the North Rona (59◦06'N, 05◦50'W) and the Isle of May (56◦11'N, 02◦33'W) breeding sites are colonies that have long standing research projects monitoring them. Plasma and milk samples were taken on North Rona and the Isle of May from 2009 until 2011, and behavioural observations were also taken during this time on North Rona. Both sites were utilised for manipulation experiments with free ranging manipulations done on North Rona from 2010 to 2011. Free ranging and pen trial manipulations were performed on the Isle of May from 2010 until 2012. Additional work to generate long term sample sets and to perfect methodologies used in the field was carried out at the SMRU captive facility at the University of St Andrews, Scotland from 2011 until 2012. Plsama samples from species other than grey seals were collected opportunistically throughout the duration of the PhD when other projects were capturing and drawing blood from individuals of interest. Both Harbour seal and Weddell seal plasma were collected via such collaborations.

2.2.1. North Rona

North Rona is a remote island in the Outer Hebrides of Scotland, 44 miles north east of the Butt of Lewis in the north Atlantic. It is a small island with shorelines ranging from rocky boulder gullies to steep cliffs 108 meters high, making access to the island

by boat difficult. The island is uninhabited and currently managed by Scottish National Heritage as a natural nature reserve due to the island's importance as a sea bird and grey seal breeding site. Grey seals were first studied on the island in 1938 by Fraser Darling (Darling 1939), and research on the breeding colony has been carried out almost every year from the 1950s until present day (Boyd et al 1971, Anderson et al 1975, Pomeroy et al 2005 and unpublished data). Grey seals are present on the island in the summer and winter, but the breeding season occurs from mid September until mid November and this is when the largest aggregations of seals occur over the grassy plains and rocky shores of the island. The size of the colony has declined over the time period it has been studied; from 1959 to 1968 the mean number of pups born per year was 2250 (Boyd and Campbell 1971), from 2000 to 2010 this number had dropped to 854 (SMRU unpublished data 2013).

The topography of the island makes it ideal for conducting long term behavioural observation studies on the main part of the colony, situated on the south part of the Fianuis peninsula in the shadow of Toa Rona and Fank Brae (Figure 2-1.). Wooden hides are erected on the top of Fank Brae looking down over the main colony, enabling researchers to record data on multiple animals from one position. Certain females in the colony are also caught twice during their stay as part of a long term project to monitor net lifetime reproductive success and health of the animals. This allowed samples to be obtained from both mothers (milk and plasma) and pups (plasma) during early and late lactation, which could then be analysed with the behavioural observation data taken around catching events. The island has no electricity, and power to run freezers must be generated by a portable generator, making samples storage difficult. By using a large portable freezer, large ice packs and a rigorous generator schedule, samples were kept frozen without defrosting through out the six week study periods.

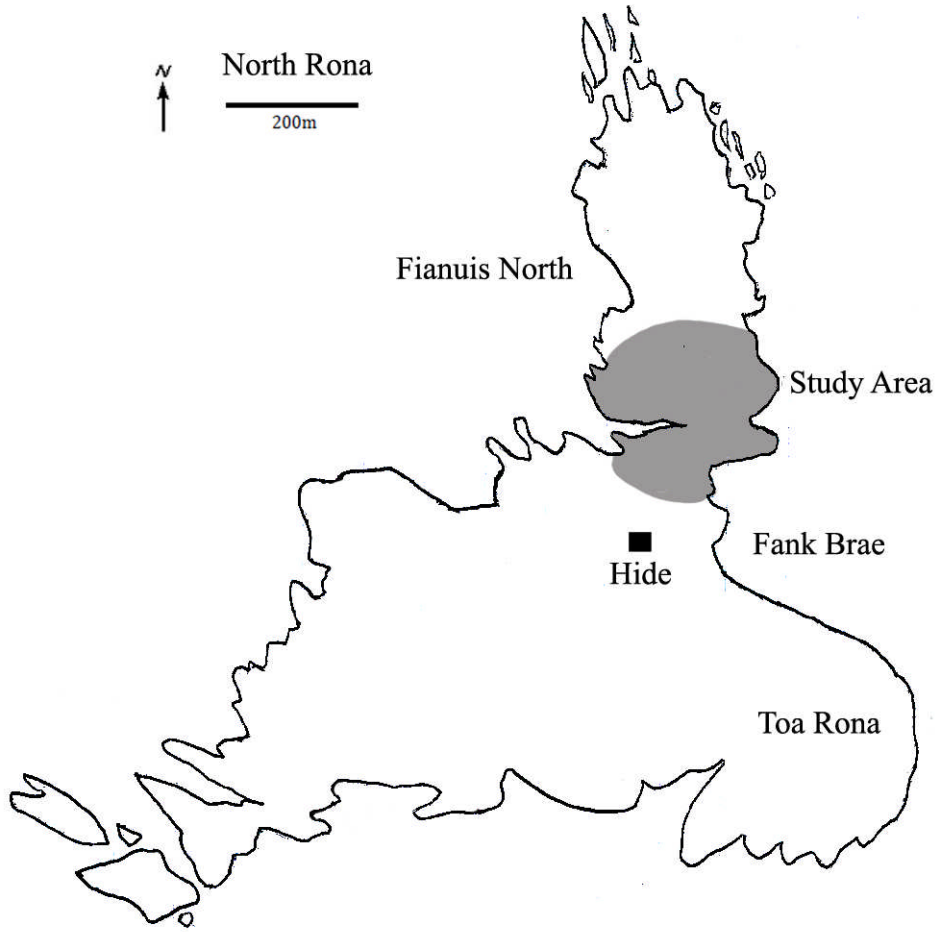


Figure 2-1 Map of North Rona, with the hide location (■) and study area containing breeding grey seals shown in grey.

2.2.2. Isle of May

The Isle of May is another offshore seal colony, located in the Firth of Forth, five miles from the east coast of Scotland. This island, like North Rona, is managed by Scottish National Heritage as a natural nature reserve due to the large sea bird and seal breeding colonies there. The island has no permanent residents but researchers are present almost year round, studying either the birds from spring to late summer or seals from autumn to winter. The island is accessible to the public during the summer but closed during the seal breeding season from mid October to late December as seals occupy most of the island during this time. There were no records of grey seals breeding on the Isle of May until the 1950s (Baker and Baker 1988), and by 1977 the

island had become an important colony for the seals, with the number of pups born annually increasing from 615 in 1982 (Brockie 1984) to 1968 in 1998 before stabilising over 2000 - 2010 with a mean of 1955 pups per year (SMRU unpublished data 2013). It is thought this rapid increase in the colony's early years was due to seals breeding on the Farne Isles being discouraged from that site and moving to this one (Brockie 1984). The colony is not concentrated in one part of the island as on North Rona, making behavioural observations on the study females that are sampled difficult. Therefore this island was used to collect a large sample set of female and pup plasma to compare with findings from North Rona. As this island does have electricity, storing samples without defrosting is far easier. Therefore the Isle of May was the site of most of the manipulation experiments that required multiple blood samples from one animal. Due to its proximity to the mainland and the relative ease of accessing the island by boat, this site was also used for all pen trial experiments as the time spent on the colony by observers could be extended if needed.

2.2.3. The Sea Mammal Research Unit Captive Facility

SMRU is located at the Scottish Oceans Institute, St Andrews and has a large Home Office licensed captive facility where up to six harbour or grey seals can be kept for up to a year. These animals are wild caught, often from nearby haul out or breeding sites, and brought into the laboratory to allow detailed study of tag design, respiration physiology, cognitive skills and learning abilities and a variety of other studies that would not be possible using wild free ranging animals. Animals are housed in outdoor seawater pools at ambient temperature and are fed a variety of fish native to Scottish waters. This study utilised five such individuals held at the facility:

Z - Adult male harbour seal caught from the Eden River and kept from 10.02.11 until November 2011.

V – Two year old male harbour seal caught from the Moray Firth and kept from 01.10.10 until 29.10.11.

W – One year old female harbour seal caught from the Moray Firth and kept from 01.10.10 until 29.10.11.

Y – Weaned male grey seal pup caught at approximately one month of age from the Isle of May and kept from 06.12.11 until December 2012.

A - Weaned female grey seal pup caught at approximately one month of age from the Isle of May and kept from 06.12.11 until November 2012.

2.2.4. Opportunistic Sampling

To gain additional baseline data on the hormone of interest, any opportunity to gain data on non-breeding adults was utilised. Several projects based at or associated with SMRU were capturing animals and sampling plasma from a variety of different phocid species during the time of this project. Basal sample sets were collected from 23 harbour seals during three separate tagging events in Scotland during 2010 and 2011. The majority (16) were taken on Shetland in August 2010, five were taken in February 2011 on the Eden River haul out in Fife and the remaining two were taken in May 2011 from the Sheildaig haul out site on the west coast of Scotland. Basal sample sets were also collected from 23 non-breeding Weddell seals during a tagging project with the British Antarctic Survey (BAS) in the Antarctic in 2011.

2.3. Behavioural Observations

For all behavioural observations taken on North Rona, the following ethogram was used to record data from mother-pup focal pairs for one to two days in both early and late lactation. Observations were carried out on two mum-pup pairs for nine hours every day using scans every 30 seconds for 15 minutes, followed by a five minute break to record other data from the colony, including the distance between the focal mothers and their dependant pups, estimated by eye, in adult seal body lengths. Both adult and weaned pup measures of one body length are used for different experiments in this thesis, please see individual chapters for more details. These metrics are defined as:

1 adult body length ≈ 2 metres
1 weaned pup body length ≈ 1 metre

Separate data was gathered for each mother and pup, giving a total of four individuals observed across a single day. This generated on average 792 scans per mum-pup pair per day, and all study females had both early and late lactation observation sessions to match with the early and late lactation blood samples taken. As a minimum of 180

scans per individual is thought to be required to provide an accurate representation of their behaviour (Twiss 1991, Culloch 2012), this scan sampling regime would generate suitable quantities of scans per individual for analysis. Observations on manipulation trials and video decoding of trials had their own protocol for recording data but used the same ethogram detailed below. Please see individual experiment chapters for further details. This ethogram was based on the behavioural categories used in previous male grey seal behaviour studies (Twiss 1991, Twiss *et al.* 1998, Twiss and Franklin 2010) with additional categories for mother and pup specific behaviours such as the ones documented in Haller *et al.* (1996). The biggest ethogram differences lie in the number of pup specific behavioural categories, which allows the inclusion of details of pup driven interaction with the mother (such as PNOS or INM) in the data.

General Behaviour:

Rest (individual stationary, with head down)	RES
Head up rest (individual stationary, with head up but neck relaxed, not alert or looking at surroundings or a specific individual)	HUR
Alert (head up, looking around or directly at something excluding the dependant pup)	ALE
Locomotion (travelling more than two body lengths from initial position)	LOC
Comfort movement (small scratching or rubbing movements)	CM
Out of Sight	OOS
Other (not specified in this ethogram)	OTH

Aggression:

Rapid sequence of aggressive behaviour, cannot see/record individual behaviours as described below	SCRAP
Open Mouth Threat (at a female)	OMTF
Open Mouth Threat (at a male)	OMTM
Aggressive Flippering (waving fore flipper at another individual, with or without physical contact)	FLPAG
Lunging (attempt to bite without making physical contact)	LUN
Bite (physical contact made)	BIT
Chase any other individual (including other sp.)	CHA

Flee from any other individual (including other sp.)	FLE
Vocalisation (recording vocalisations potentially confounded by wind and distance from the hide)	VOC

Female behaviours:

Flippering (gentle rubbing of pup's head or back to encourage suckling)	FLP
Present nipples to pup	PRE
Nursing	SUC
Check pup (same as ALE but directed specifically at the pup, without any physical contact)	CKP
Interact with pup (physically touching the pup, with or without the pup reciprocating)	INP
Birth	BIR
Copulation, successful	COP
Attempted copulation	COPAT
Failed copulation	COPFA

Pup behaviours:

Pup nosing female (to encourage nursing)	PNOS
Suckling	SUC
Possibly suckling but out of sight	SUC?
Check mother (same as ALE but directed specifically at the mother, without any physical contact)	CKM
Exploration, general nosing around the soil, plants and rocks in the immediate vicinity	EXP
Play, rolling around or chewing flippers	PLY
Interact with mum (physical contact with the mother, with or without the mother reciprocating, includes all interactions except PNOS)	INM
Thrown by another individual	THR
Bitten by another individual	BTN
Dead	D
Eaten (can be alive or dead, usually from gulls)	EAT

2.4. Capture Protocol, Sampling and Storage

Wild grey and harbour seals are regularly captured for sample collection for a variety of projects at SMRU. All plasma samples from adult individuals were taken using the same methodology, while the protocol for juveniles, weaners and pups was slightly different (outlined below). The protocol for treatment and storage of samples after collection was the same across all species and age groups.

2.4.1. Adult animals – Grey Seals

Adult grey seals sampled for this study were all breeding females on either North Rona or the Isle of May colonies. The majority were 'study females' with a history of pupping on the colony and a record of prior captures and data. Mothers and their dependant pups were caught twice in one season, once 2-4 days after birth and then approximately 10 days later in late lactation. Mothers were approached and administered 1ml/kg of Zoletil® (Virbac) intramuscularly using blow darts, and were then left for ten minutes to allow the drug to take effect. Mothers were then restrained in a net for sampling as the drug would not render them fully unconscious, and their pups were caught and restrained manually without drugs. Plasma was drawn into heparin vacutainers (unless otherwise stated in the methods of individual experiments) from the extradural vein and samples were immediately put on ice until they could be spun and frozen at the end of the day. Milk samples were obtained by injecting 1ml of oxytocin (Oxytocin-S, Intervet UK Ltd) intravenously to stimulate milk let down. Samples were then collected by vacuum pump and put on ice immediately until they could be frozen. Obtaining milk samples without oxytocin injection was extremely difficult. Only two samples out of 250 were obtained without the use of the drug. These were used to allow detection of any interference of the injection with the natural levels in the milk. Mothers were monitored through out the capture period for signs of respiratory distress, and if this occurred the individual was intubated and given Dopram® (Phzer) to resuscitate them. Mothers coming out of the drugged state early were given additional intravenous Zoletil® if required. Pups were replaced with mothers as soon in the capture procedure as possible to minimise the risk of the pair becoming separated after the capture. All mothers were left to recover naturally with their pups; no reversal was used on any individual. Mothers typically regained

motility and awareness rapidly as Zoletil® induces short duration anaesthesia lasting only approximately 20 minutes.

2.4.2. Adult animals – Other Species

The protocol for taking a plasma sample in other species in this study was the same as outlined above, with differences in the methods used to capture and induce anaesthesia. Harbour seals caught on haul out locations were approached rapidly in boats and captured when they attempted to go to sea using a pop-up net deployed just offshore, which was placed there earlier in the week. The net was then pulled onto land and individual seals restrained in hoop nets until they could be drugged using an intravenous injection of Zoletil® into the extradural vein. Individual Weddell seals were approached on ice floe haul outs, darted and caught before they could re-enter the water.

2.4.3. Juveniles, Weaners and Pups

The protocol for taking plasma samples from non-adult individuals differed from that for adults in the methods used to capture them, the use of drugs to chemically restrain individuals and the site from which the plasma sample was drawn. For non-adult individuals, the sampling procedure used was determined by their age. Animals over eight months of age were physically restrained and then chemically immobilised using an intravenous injection of Zoletil® for plasma sampling. Individuals younger than this were categorized as either ‘weaners’ (pups from the current breeding season that had not been seen with a mother for two consecutive days, generally over 20 days of age) or ‘pups’ (still dependant on their mothers and suckling, generally under 20 days old). Both of these groups received no drugs during sampling (unless otherwise specified in the methods of individual experiments) and were physically restrained for sampling. Most plasma samples were drawn from the extradural vein but for some individual’s samples were taken from the plantar network of veins on the ventral surface of the hind flippers.

2.4.4. Plasma Collection, Processing and Storage

All plasma samples were kept on ice or chilled using ice packs until they could be brought back to a laboratory environment for processing and storage. Oxytocin has a short half life in biological mediums (Kowalski *et al.* 1998) and it is recommended

that samples are kept chilled until they can be frozen (C.S. Carter 2010 pers. comm). The enzyme-linked immunosorbent assay (ELISA) protocol for the kit (Assay Designs Inc, Ann Arbor, MI, USA) used in this study recommends the use of Ethylenediaminetetraacetic acid (EDTA) vacutainers, the addition of Aprotonin to the plasma after collection and storage at -70°C to ensure preservation of all oxytocin in the samples. This study did not use Aprotonin due to difficulties in obtaining the now discontinued substance, but used heparin vacutainers and froze samples at -20ºC (C.S. Carter 2010 pers. comm). Please see Chapter 3 for the experiments investigating and justifying these deviations from the recommended protocol.

Samples taken at the SMRU captive facility were put on ice immediately and then spun, aliquoted and frozen within half an hour of collection. Samples taken in the field were placed immediately in a cool box with multiple ice packs to keep them chilled, and were kept this way until the team returned to the laboratory at the end of the day. They were then spun immediately, aliquoted and frozen until they could be transported back to the main laboratory at SMRU for analysis.

2.5. Extraction and ELISA Protocol

2.5.1. Plasma Sample Extraction

The majority of plasma samples collected in this study contained natural concentrations of oxytocin, which when detected and quantified would generate a basal dataset of this hormone in phocid seals. A subset of samples generated were part of clearance rate and artificial manipulation trials, and therefore the protocol for their analysis had to be adapted. Please see the methods section of these individual experiments in Chapters 3, 6 and 7, which details the specific adaptations of the protocol to the higher, unnaturally generated oxytocin levels in these trials.

All plasma samples taken from mother/pup pairs on North Rona 2010 and 2011, from harbour seals and from weaned pups and yearlings on all colonies for all seasons were extracted from 3ml of plasma. 3ml was used rather then 1ml because no oxytocin analysis has ever been done in phocid seals before, and basal oxytocin concentrations in extracted plasma of other species are typically so low they are close to undetectable (Kowalski *et al.* 1998). Therefore initially this study used a high volume of plasma to extract the hormone from, and by concentrating 3ml worth of oxytocin into a 200µl volume, accurate detection was far more likely. Once it was established that the mean

basal level of phocid oxytocin was high enough to allow extraction from 1.5ml of plasma, all samples still to be analysed (Isle of May mother/pup 2010 and 2011 and Weddell seal samples) were extracted from 1.5ml.

The extraction protocol given with the Assay Designs Oxytocin ELISA (Assay Designs Inc, Ann Arbor, MI, USA) was followed with some alterations to overcome problems with plasma samples blocking the Sep-Pak columns (Waters Corporation, MA, USA) used to extract the peptide due to the high lipid and albumin concentrations present in phocid plasma (Hall 1998). Different protocols for 3ml and 1.5ml extraction exist because the Sep-Pak columns have a limit of 3ml of sample volume they can extract accurately, and the 3ml protocol exceeds this by generating 6mls of acidified plasma during the extraction procedure. The protocols for 3ml and 1.5ml extractions are as follows:

3ml extraction protocol

1. Defrost 3ml of plasma for 30 minutes at room temperature
2. Add an equal volume of 0.1% trifluoroacetic acid (TFA) in deionised water to the sample, giving 6ml of solution. Aliquot into three 2ml eppendorfs.
3. Centrifuge at 17,000 x g for 30 minutes.
4. Equilibrate a 200mg C18 Sep-Pak column with 1ml acetonitrile and then 15ml of 0.1% TFA in water and discard the wash. Pump the solutions through at 1ml/30 seconds.
5. Load half (3ml) of the acidified plasma sample onto the column, at 1ml/minute.
6. Wash with 10ml of 0.1% TFA in water at 1ml/minute and discard the wash.
7. Elute the sample by applying 3ml of 60:40 ratio acetonitrile:0.1%TFA in water solution at 1ml/minute. Collect the eluant, which can be kept refrigerated for up to 24 hours.
8. To extract the other half of the sample, equilibrate another Sep-pak column and repeat steps 4 – 7 to generate another 3ml sample.
9. Evaporate one of the final tubes to dryness using a centrifugal concentrator under vacuum for 7 hours.
10. Pipette all of the contents of the remaining tube into the dry tube, re-filling it with the other half of the sample.
11. Evaporate to dryness again for 12 hours.

12. Once both of the 3ml samples have been dried down into one tube, samples can be frozen at -20ºC until ELISA.

1.5ml extraction protocol

This is exactly the same as the 3ml protocol except a second extract does not need to be generated and only one evaporation to dryness cycle needs to take place.

1. Defrost 1.5ml of plasma
2. Add an equal volume of 0.1% trifluoroacetic acid (TFA) in water to the sample, giving 3ml of solution.
3. Centrifuge at 17,000 x g for 30 minutes.
4. Equilibrate a 200mg C18 Sep-Pak column with 1ml acetonitrile and then 15ml of 0.1% TFA in water and discard the wash. Pump the solutions through at 1ml/30 seconds.
5. Load the 3ml acidified plasma sample onto the column, at 1ml/minute.
6. Wash with 10ml of 0.1% TFA in water at 1ml/minute and discard the wash.
7. Elute the sample by applying 3ml of 60:40 ratio acetonitrile:0.1%TFA in water solution at 1ml/minute. Collect the eluant, which can be kept refrigerated for up to 24 hours.
8. Evaporate to dryness using a centrifugal concentrator under vacuum for 7 hours.
9. Freeze at -20ºC until ELISA.

Please note that when extracting multiple samples in one day, all acidified plasma and final sample tubes must be chilled, either by placement on ice or in a refrigerator. Additional centrifuging of acidified plasma samples is also needed throughout the day as the time since the first 30 minute spin lengthens to prevent samples blocking the Sep-Pak columns. In the above protocols, samples were re-spun for 15 minutes 1 hour after first spin, then every 2 hours after the last spin to prevent blocking of Sep-Pak columns.

2.5.2. Milk Sample Extraction

The protocol given with the Assay Designs Oxytocin ELISA does contain instructions for analysing milk samples. This was followed with the addition of peptide extraction at the end of the clarification procedure. Grey seal milk contains a high proportion of

fat, containing up to 60% fat across the lactation period (Lydersen *et al.* 1995). This must be removed from the sample prior to analysis otherwise the high lipid content has the potential to interfere with the ELISA procedure and the oxytocin concentration detected.

1. Defrost 3ml of milk for 30 minutes at room temperature.
2. Centrifuge the sample at 10,0000 rcf for 15 minutes.
3. Using a syringe or pipette carefully pierce through the top layer of fat and collect the lower layer of supernatant.
4. Centrifuge the supernatant again at 10,0000 rcf for 15 minutes.
5. Collect the lower layer of supernatant once more and centrifuge a final time at 10,0000 rcf for 15 minutes.
6. Collect the lower layer of supernatant for extraction, the volume will have decreased to around 1ml but this will vary across samples. If the resulting volume is greater than 1.5ml, the acidified sample will have to be split and two extractions done (see plasma extraction protocol).
7. Add an equal volume of 0.1% trifluoroacetic acid (TFA) in water to the sample. Centrifuge at 17,000 x g for 30 minutes.
8. Equilibrate a 200mg C18 Sep-Pak column with 1ml acetonitrile and then 15ml of 0.1% TFA in water and discard the wash. Pump the solutions through at 1ml/30 seconds.
9. Load the acidified sample onto the column, at 1ml/minute.
10. Wash with 10ml of 0.1% TFA in water at 1ml/minute and discard the wash.
11. Elute the sample by applying 3ml of 60:40 ratio acetonitrile:0.1%TFA in water solution at 1ml/minute. Collect the eluant, which can be kept refrigerated for up to 24 hours.
12. Evaporate the sample to dryness using a centrifugal concentrator under vacuum for 7 hours.
13. Once dried down, samples can be frozen at -20ºC until ELISA.

2.5.3. ELISA

Several commercial ELISA and radioimmunoassay (RIA) kits are available to buy for oxytocin analysis. All available kits were explored as possibilities for this research, and the Assay Designs oxytocin ELISA kit was selected as the most suited to this

study. Please see Chapter 3 for the experiments investigating each of the kits and justification for choosing the Assay Designs kit. All sample analysis in this study were carried out using Assay Designs kit apart from those involved in testing the different analytical kits available to purchase.

The Assay Designs ELISA is a competitive immunoassay which uses a polyclonal antibody to bind any oxytocin in a sample to the wells on a plate. After the addition of the sample, an oxytocin conjugate containing oxytocin bound to alkaline phosphatase is also added to bind to any exposed antibodies left in the wells. The plate is then incubated for 24 hours, the reagents washed away and a substrate is added that reacts to the alkaline phosphatase, p-nitrophenylphosphate (pNpp solution), to produce a yellow colour. The greater the intensity of the yellow colour in a well, the less oxytocin is present in a sample (Figures 2-2 and 2-3).

The protocol given with the ELISA kits was followed with one alteration; a 750pg/ml point on the standard curve was added to allow greater accuracy in detecting high oxytocin concentrations. All plates were run with a standard plasma sample run in duplicate from a plasma pool to allow calculation of inter and intra plate covariance. Intra-assay coefficient of variance for this assay was 4.6% and inter-assay coefficient of variance over the 40 plates used in this thesis was 8.3%. Recovery rates for the extraction and ELISA procedure were 107.2% (n=10). Recovery rates were calculated using stripped grey seal plasma spiked with known quantities of oxytocin, then extracted and analysed using the ELISA kit. Several different re-hydration and dilution factors were used to run samples with suspected extremes of high or low oxytocin in them (please see the methods section of individual chapters for details of what was used for specific experiments).

Plates were read using a BioTek ELx800 reader and the standard curve and assay results for all plates were then calculated using the calibFit package (Haaland *et al.* 2011) in R version 2.9.2 (R Development Core Team, 2008)

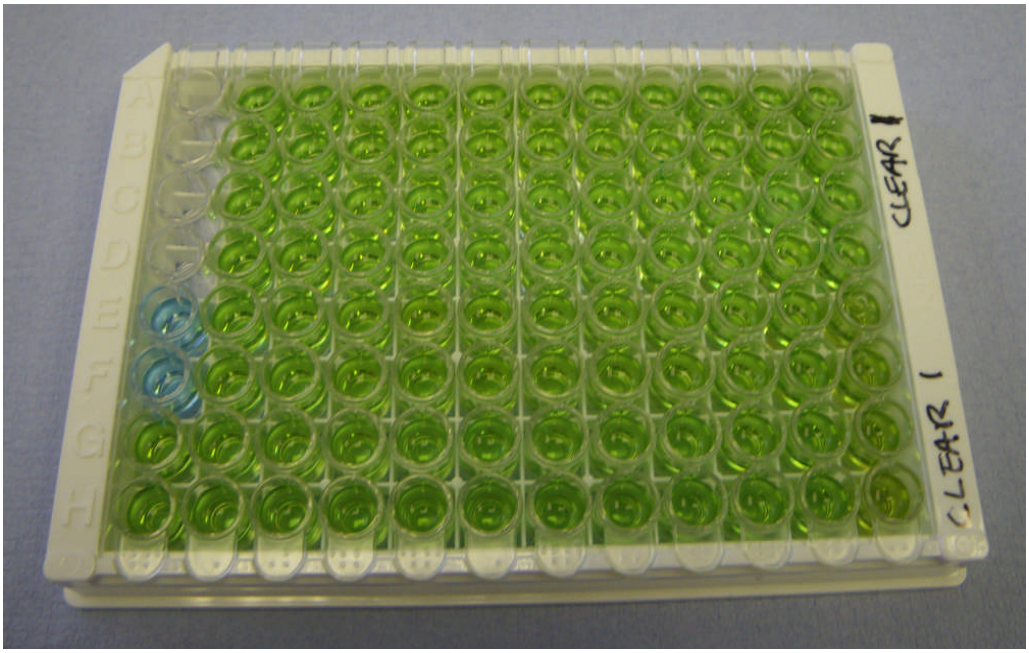


Figure 2-2. ELISA plate loaded with samples and oxytocin conjugate, ready for incubation for 24 hours.

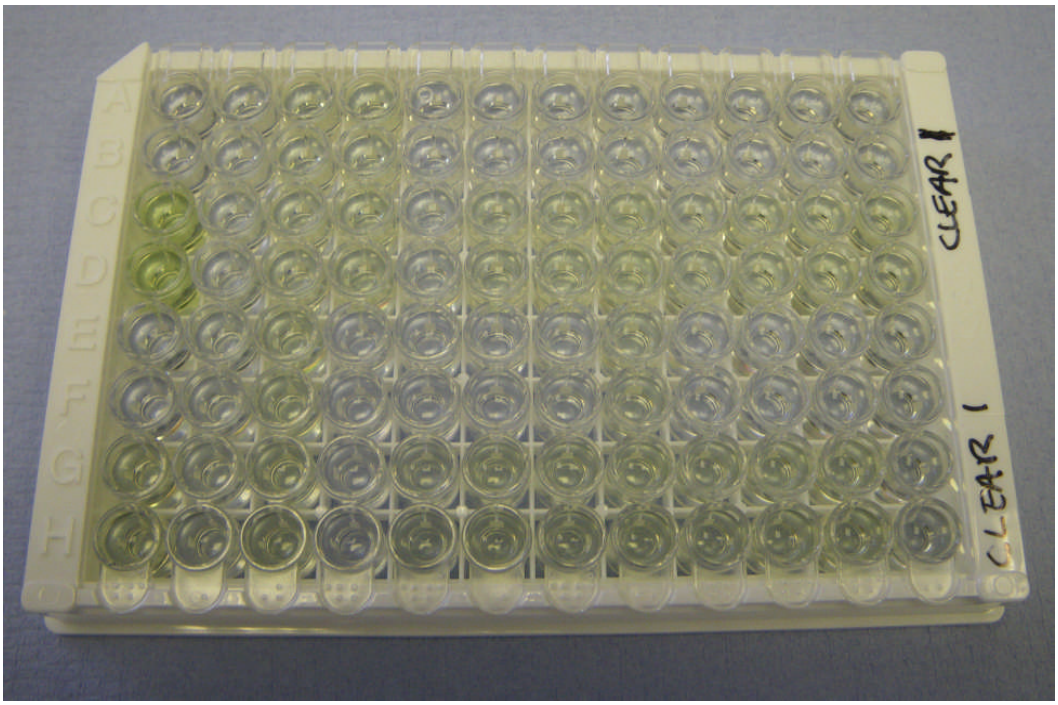


Figure 2-3. ELISA plate ready to be analysed on a plate reader, after washing and addition of pNpp solution. This plate contains samples from a clearance trial which generated samples with very high oxytocin concentrations. Samples on this plate are therefore run undiluted and at x100 dilution to ensure they fit on the standard curve. The differences between the undiluted samples (clear) and diluted samples (yellow colour) are obvious.

2.6. Statistical Analysis

All analyses were performed using the statistical package R 2.15.0 (R Development Core Team, 2012). A variety of packages were used through out this thesis, please see individual chapters for details.

Chapter 3: Measuring Oxytocin

3.1. Introduction

Before any samples taken for this project can be analysed and interpreted with confidence, a reliable and accurate assay for oxytocin must be in place. Studies looking to investigate peripheral levels of the hormone have existed since the 1950s (Ginsburg and Smith, 1959) and have become popular in the 21st century. Oxytocin has been assayed in various substrates including saliva (Carter *et al.* 2007, Feldman *et al.* 2010a and. 2010b, Weisman *et al.* 2012a and 2012b), urine (Nagasawa *et al.* 2009, Moscovice and Ziegler 2012, Crockford *et al.* 2013), cerebro-spinal fluid (Devarajan and Rusak, 2004, Martínez-Lorenzana *et al.* 2008), and milk (Leake *et al.* 1981, Prakash *et al.* 2009). However, the most common medium used for oxytocin detection is plasma (of 50 papers reviewed using one or multiple substrates, n=39 plasma, n=9 saliva, n=8 urine, n=6 cerebro-spinal fluid, n=5 serum and n=2 milk). While no study has detected the hormone in a pinniped species before, oxytocin is a widely conserved hormone across mammals (Gimpl and Fahrenholz 2001) and prior studies have successfully analysed plasma oxytocin levels in humans (Dawood *et al.* 1981, van der Post *et al.* 1997, Strathean *et al.* 2009), sheep (Sheldrick and Flint 1981, Da Costa *et al.* 1996), rodents (Devarajan and Rusak 2004, Carter *et al.* 2007, Martinez-Lorenzana *et al.* 2008) dogs (Odendaal and Meintjes 2003) and primates (Amico *et al.* 1990, Maestripieri *et al.* 2009, Moscovice and Ziegler 2012, Crockford *et al.* 2013). Developing a protocol for detecting pinniped oxytocin using existing analysis kits should therefore be possible, despite peculiarities in phocid plasma (Hall 1998) that may interfere with the assays.

There are a variety of techniques that can be used to analyse oxytocin levels in mammals, including RIA (Strathean *et al.* 2009) ELISA (Martinez-Lorenzana *et al.* 2008) high performance liquid chromatography (HPLC) (Dudkiewicz-Wilczynska *et al.* 2000) and other experimental quantification methods such as surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (SELDI-TOF MS) (Cool and DeBrosse 2003). In this chapter commercially available assay kits will be tested against each other to determine the most sensitive and accurate way to analyse samples for all studies that follow in this thesis.

Despite the increasing number of studies using plasma to investigate oxytocin, there is still contention about the appropriate way to collect and analyse samples accurately, and there has been a call for new research into reliable, accurate methodologies for investigators to utilise in future research (McCullough *et al.* 2013). Such contention in the literature makes selection of the most appropriate protocol difficult. The majority of studies published use an ELISA kit manufactured by Assay Designs Inc (Ann Arbor, MI, USA). However, despite this manufacturer providing a protocol for collection and preparation prior to analysis, there is huge variation between studies in the methods researchers actually employ (Table 3-1.).

Table 3-1. Variety of analysis protocols in 39 different published studies on oxytocin concentrations in blood.

Extracted Plasma		Vacutainer Choice		Inhibitor Use	
Yes	13	EDTA	12	Yes	7
No	17	Heparin	10	No	7
Not Specified/Serum	9	Not Specified	17	Not Specified	25

In addition to the varying analysis protocols, there is disagreement in the literature over whether oxytocin concentrations measured in raw plasma are correlated with those in extracted samples, and how the two are related. Currently some studies rely on the hypothesis that there is a direct relationship between oxytocin concentrations determined in extracted and raw samples (Hoge *et al.*, 2012). Michopoulos *et al.* (2011) provide data showing a correlation between oxytocin levels in raw and extracted serum samples and Robinson (1980) developed an assay protocol utilising a RIA to accurately detect oxytocin in raw plasma samples. However, two papers to date have directly tested the benefits of extracting plasma samples prior to analysis, with both recommending the use of solid phase extraction with C18 Sep-Pak columns (Bachem, San Carlos, CA) as the best method to gain accurate detection levels for oxytocin in plasma (Szeto *et al.* 2011, Cool and DeBrosse 2003). Szeto *et al.* (2011) go on to report no correlation between oxytocin concentrations measured in raw plasma and those in extracted plasma, and call for further work to be carried out in order to develop reliable methods for oxytocin analysis. In this chapter, we use a longitudinal sample set to investigate the effect of extraction, vacutainer type, freezer

temperature and use of inhibitor on oxytocin concentrations detected in plasma samples.

In addition to developing an accurate assay for this hormone specifically in phocid seals, it is vital to ensure that abnormal concentrations of oxytocin are not being generated during the capture and sampling procedure. The relationship between oxytocin and cortisol is not fully understood, but manipulation studies injecting either of the hormones have found that oxytocin plays a role in the regulation of stress responses (Devarajan and Rusak 2004). Oxytocin infusions prior to a stressor was found to lower the cortisol response in sheep (Cook 1997) and humans (Ditzen *et al.* 2009), oxytocin injections consistently decreases corticotrope function levels in humans (Legros *et al.* 1984 and 1987) and cortisol injections cause increases in plasma oxytocin levels (Tops *et al.* 2012). Additionally, oxytocin is documented to be released during extreme stressors such as restraint over an extended time period in rodents (Grippo *et al.* 2009, Pournajafi and Carter, unpublished observations) and is hypothesised to occur during procedures such as injections (Devarajan and Rusak 2004). Therefore any attempt to study basal levels of this hormone in seals, which must be manually or chemically restrained during the sampling procedure, must be accompanied by investigation of whether oxytocin is released when sampling occurs.

The objectives of this chapter were:

1. To test the accuracy and suitability of the three commercially available oxytocin assay kits for use analysing phocid plasma samples.
2. To investigate the effect of extraction, vacutainer choice, storage temperature and the use of an inhibitor on the concentration of oxytocin detected in plasma samples.
3. To explore the relationship between raw and extracted oxytocin concentrations, and determine whether one can be used to accurately predict the other.
4. To quantify any elevation in oxytocin that may occur from rising cortisol levels due to capture and handling stress.
5. To quantify the concentrations of oxytocin in phocid milk.

3.2. Methods

3.2.1. Assay selection for the detection of plasma oxytocin concentrations in phocid seals

Study animals

Samples from 37 female grey seals from the Isle of May breeding colony in 2009 and 15 mothers and pups from 2010 where used in this study. All females had dependant pups at time of sampling.

Plasma sampling and analysis

The protocol described in Chapter 2 was used to capture and restrain seals for sampling. There were only three commercially available assay kits for oxytocin analysis available at the time of publication of this thesis, and all were tested with seal plasma to determine the best one to use for the analysis of all subsequent samples for this thesis. An RIA and ELISA from Phoenix Pharmaceuticals (Phoenix Pharmaceuticals Inc, CA, USA) were first used comparing the concentrations detected in the same 31 females from the 2009 season on the Isle of May. For these assays, oxytocin was extracted prior to analysis from 1ml of plasma as directed in the protocol accompanying the kits. On the ELISA several samples were also run without extraction to allow discrimination between a failure of the assay to detect seal oxytocin and a failure in the extraction protocol to successfully isolate it.

An ELISA from Assay Designs (see Chapter 2.) was then tested using 24 of the same females as tested in the Phoenix Pharmaceuticals experiments and seven additional females from 2009. The protocol for the ELISA was followed exactly with one amendment based on the results of the Phoenix Pharmaceuticals assays; oxytocin was extracted from 3ml rather than 1ml of plasma. Finally the Assay designs kit was used to analyse 15 females/pup pairs from the 2010 season on the Isle of May, using oxytocin extracted from 1.5ml of plasma.

3.2.2. Effects of sampling and analysis protocol on plasma oxytocin concentrations

Study animals

Three seals provided blood samples for the comparison of vacutainer type and extraction protocol. Samples were collected over the period the seals were within the SMRU captive facility. Individuals Z (adult male harbour seal), Y (male grey seal kept from weaning until 1 year) and A (female grey seal kept from weaning until 1 year) where used for this study.

Plasma sampling and analysis

Plasma samples were taken at approximately monthly intervals throughout the period of captivity (n=27). A sample collected using heparin vacutainers was collected in all 27 sampling opportunities; however during two of the sampling opportunities it was not possible additionally to collect an EDTA sample, giving 25 matching EDTA plasma samples and a total number of 52 samples to compare to each other. The protocol outlined in Chapter 2. was used to capture and restrain seals for sampling. Samples were drawn from the extradural vein into both 10ml lithium heparin and EDTA vacutainers without addition of aprotinin and stored on ice until they could be spun and frozen at -20ºC. At each sampling event four vacutainers of plasma (two of each type) were taken. Plasma was then extracted and analysed for oxytocin concentrations using the protocol described in Chapter 2. Both raw plasma and extracted plasma were analysed for all 52 samples collected during analysis, giving a total sample size of 104 samples for this study. Intra-assay coefficient of variance for this assay was 4.6% and inter-assay coefficient of variance over the seven plates used in this study was 4%.

Statistical analysis

Generalised additive mixed models (GAM) (Wood, 2006a) were used to take into account variability in oxytocin generated by species, sex, the day of year the samples were taken, different vacutainer type and extraction protocol. The models had a gamma error distribution with a log link and were fitted using the multiple generalized cross validation library mgcv (Wood, 2012). As any variation with ‘day of year’ (DOY) in unlikely to be linear, this was fitted as a smooth function (Wood,

2006b). Differences between species and sex in the seasonal pattern were examined by fitting additional smooth functions. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). All other variables were fitted as fixed effects. Biologically plausible first order interactions such as "tube type*extraction protocol" were also considered. Backwards stepwise elimination was carried out by using the Akaike information criterion (AIC) to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models (Richards 2011). On this basis, all smooths were removed from the model, and DOY was removed entirely, simplifying the model into a generalised linear model (GLM) and leaving four fixed effects and one interaction term which produced the best performing model.

3.2.3. Relationship between extracted and raw oxytocin plasma concentrations

Study animals

Individual V (male two year old harbour seal) was used to generate a dataset on the clearance rate of oxytocin in phocid seals using extracted and un-extracted samples. Additionally raw/extracted sample data generated from the above experiment (Chapter 3, 3.2.2.) with animals Z, Y and A was used to attempt to predict extracted values from raw values.

Clearance trial

For the duration of the 1 hour trial, the study animal was captured and chemically immobilised using Zoletil®. A baseline sample was drawn from the extradural vein into 10ml lithium heparin vacutainers, and then 3.96µg oxytocin (0.022ml Oxytocin-S, Intervet UK Ltd with 0.128ml saline solution) was injected intravenously to create a hormone spike in the plasma. Serial samples where then taken every minute for the first five minutes of the trial, then every five minutes until 20 minutes from injection had elapsed, and finally every ten minutes until one hour from injection. Samples were then stored, spun, frozen and analysed as described above, generating clearance curves for both extracted and raw versions of the same samples. The plasma clearance rate (CL) for both raw and extracted plasma samples from the same clearance trail were calculated using the following equation (Morin *et al.* 2008):

$$CL = D/AUC$$

Where Area Under Curve (AUC) is the area under the curve generated in the GAMs detailed below describing the relationship between plasma oxytocin concentrations and time post injection and D is the initial bolus of oxytocin injected in picograms (pg).

Statistical analysis

Non-linear and GAM approaches were investigated to quantify the clearance curves generated from the IV oxytocin injection. The GAM approach was determined to be the most suitable for comparing the quality of the two clearance curves, and GAMs were made for both extracted results and raw results. Both used one fixed effect (minutes post oxytocin injection) and as this variable was not linear it was fitted as a smooth function with smoothing parameters set by maximum likelihood. Confidence intervals and percentage deviance explained were then used to examine which dataset accurately represented the clearance of the peptide from the blood.

To investigate any correlations between oxytocin concentrations detected in extracted and raw plasma samples (n=52), two GLMs were used to model the relationship between the two measures taken with heparin (n=27) and EDTA (n=25) vacutainers separately and to predict extracted values of oxytocin from concentrations detected in raw samples using the data from animals Z, Y and A. All GLM fits were fitted with gamma error distributions with log links and were checked with diagnostic plots and AIC scores to determine the best fits for the data.

3.2.4. The effect of capture and handling stress on plasma oxytocin concentrations

Study animals

Two seals provided blood samples for the quantification of the effect of handling stress on plasma oxytocin concentration. Samples were collected over the period the seals were within the facility. Individuals Y (male grey seal kept from weaning until 1 year) and A (female grey seal kept from weaning until 1 year) where used for this study.

Plasma sampling and analysis

Sample data generated from section 3.2.2. of this chapter with animals Y and A was used for this model, as time of 1st disturbance, time of sampling and use of drug to immobilise were all recorded for these two animals.

Statistical analysis

Two separate GLMs were used to investigate any relationship between time taken to obtain a plasma sample, the use of chemical immobilisation or physical restraint and vacutainer type on the oxytocin concentrations detected in raw and extracted plasma. This could not be included in the GLM described in section 3.2.2. as some values were missing from one individual's data, which would have reduced the number of samples included in that analysis. The time in minutes it took to obtain a plasma sample from the initial disturbance to the individual, what form of restraints (physical or chemical) were used for the procedure, an interaction term for sampling time and restraint type and the type of vacutainer used were fitted as fixed effects and a gamma error distribution with a log link was used. Backwards stepwise elimination was carried out by using the AIC to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models.

3.2.5. Protocol for the detection of oxytocin concentrations in phocid milk

Study animals

Samples from four female grey seals from the Isle of May breeding colony in 2009 where used in this study. All females had dependant pups at time of sampling.

Milk sampling and analysis

The protocol described in Chapter 2. was used to capture, restrain and collect samples from seals. The Assay designs kit was used to analyse milk from four mothers, all four were run without extraction and were diluted across a six point dilution series and then run on the plate to maximise the chances of the concentration in the milk being on the detection curve for this plate. Two of the mothers sampled generated enough volume to test the extraction process using both 3ml and 1.5ml of milk. These were run on the plate in a four point dilution series.

3.3. Results

3.3.1. Assay selection for the detection of plasma oxytocin concentrations in phocid seals

Of 31 samples extracted from 1ml plasma and run on the Phoenix Pharmaceuticals RIA and ELISA, 11 were detected on the RIA and 23 on the ELISA. All other samples contained oxytocin concentrations that were too low to be detected by the assays. Mean oxytocin concentration for the RIA was 14.2pg/ml (SD: 0.11) and 11.6pg/ml (SD: 7.37) for the ELISA. Mean oxytocin detected in raw plasma on the ELISA was 167.5pg/ml (SD: 71.17). When using the Assay Designs kit and extracting from 3ml, all samples (31/31) were detected, however nine contained levels too high to be quantified on the standard curve. Mean oxytocin concentration was 6.88pg/ml (SD: 3.03). Finally when extracting from 1.5ml mother and pup plasma and using the Assay Designs kit, all samples were detected and quantifiable. A mean oxytocin concentration for the mothers was 8.28pg/ml (SD: 2.09), for pups was 19.9pg/ml (SD: 7.37) and for raw maternal plasma was 258.9pg/ml (SD: 15.39).

3.3.2. Effects of sampling and analysis protocol on plasma oxytocin concentrations

Of the variables tested, the GLM model showed that analysis protocol (n=104 GLM, $F_{3, 101} = 934.8$, $p<0.001$) sex (n=104 GLM, $F_{1, 103} = 0.011$, $p=0.01$) and species (n=104 GLM, $F_{1, 103} = 46.9$, $p<0.001$) significantly affected the oxytocin detected in samples (Table 3-2). There was no significant difference between extracted samples taken with EDTA (mean = 8.1 ±0.6pg/ml) or heparin (mean = 8.3 ±0.6pg/ml) vacutainers ($p=0.71$). However there were significant differences between all extracted and raw plasma protocols regardless of vacutainer type ($p<0.001$) and between samples analysed raw taken using EDTA (mean = 543.2 ±43.6pg/ml) and heparin (mean = 300.9 ±19.6pg/ml) vacutainers ($p<0.001$) (Figures 3-1 and 3-2).

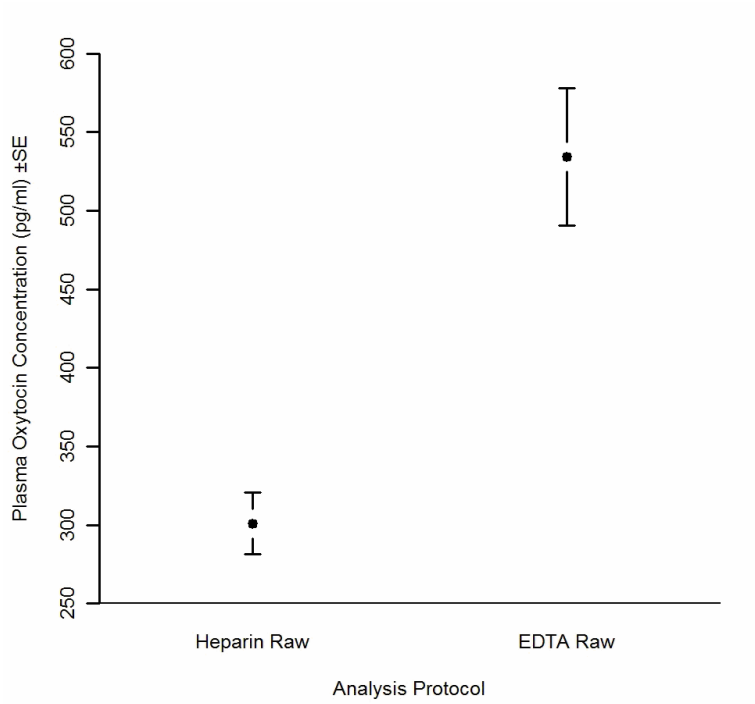


Figure 3-1. Mean concentrations of oxytocin detected in raw phocid plasma according to vacutainer type used to collect the sample with standard error bars.

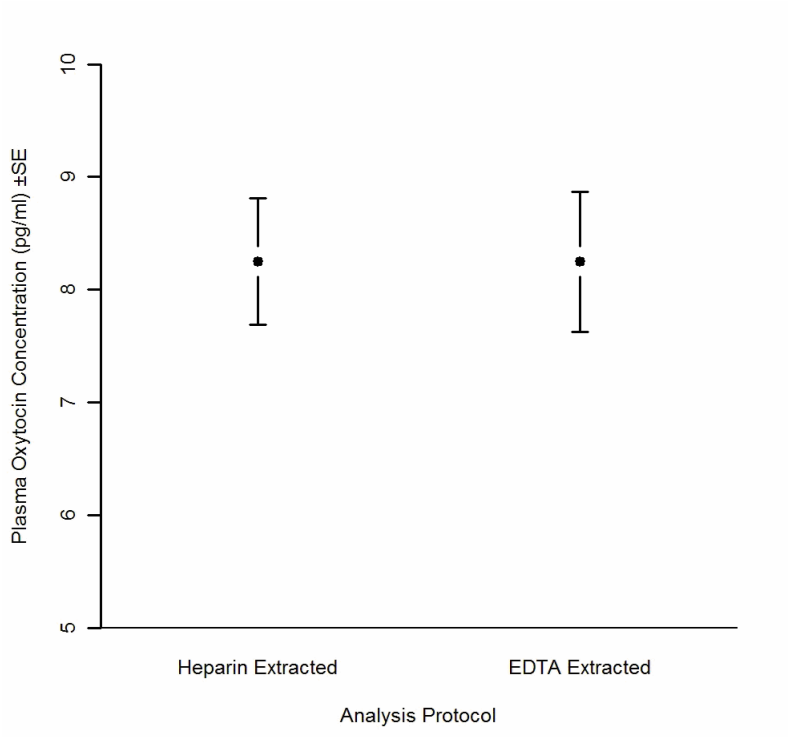


Figure 3-2. Mean concentrations of oxytocin detected in extracted phocid plasma according to vacutainer type used to collect the sample with standard error bars.

Table 3-2. Fixed effect variables from the GLM of the effect of analysis protocol on detected oxytocin concentration, their estimates, standard errors and p values.

GLM variable	Estimate	Standard Error	p-value
Analysis Protocol (Heparin Extracted)	0.03	0.087	0.71
Extraction Protocol (Heparin Raw)	3.66	0.087	<0.001
Extraction Protocol (EDTA Raw)	4.21	0.088	<0.001
Sex	-0.18	0.072	0.012
Species	-0.53	0.077	<0.001

3.3.3. Relationship between extracted and raw oxytocin plasma concentrations

Both GAM models showed that in raw and extracted plasma there was a significant decline in oxytocin over time towards the basal level (n=13 in each GAM, for both $p<0.001$). However the oxytocin values detected using raw plasma was much less successful at producing an accurate clearance curve compared to one generated with extracted plasma (percentage deviance explained, raw plasma: 30.7%, extracted plasma: 85.2%). In addition the confidence intervals were much wider around the fitted curve for the raw results (Figure 3-3). The plasma clearance rate calculated using the curve generated with extracted plasma was 0.035 L/min/kg, while the plasma clearance rate calculated using the curve generated with raw plasma was 0.004 L/min/kg.

No relationship was found between oxytocin concentrations detected in raw and extracted plasma samples taken with heparin (n=27 GLM, $F_{1, 26} = 1.24$, $p=0.25$) or EDTA vacutainers (n=25 GLM, $F_{1, 24} = 2.47$, $p=0.12$) (Figure 3-4). Furthermore, when the best model was used to generate predicted oxytocin concentrations in extracted samples from raw values, the resulting dataset (mean = 2.1 ±0.008 pg/ml) was significantly different from the true values (mean=8.2 ±0.4 pg/ml) when analysed using a Welch two sample T test ($t_{15}=8.2$, $p<0.001$).

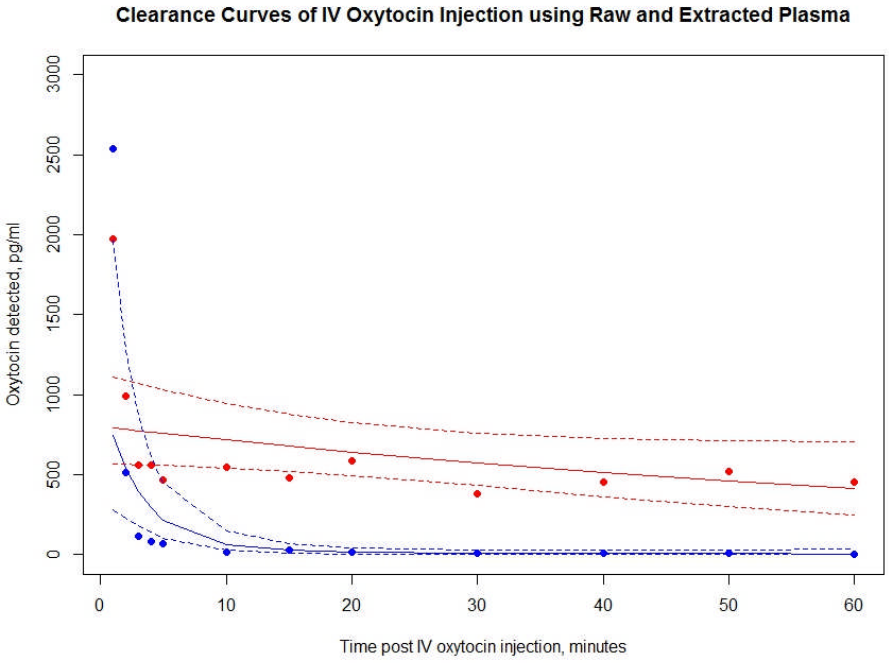


Figure 3-3. Clearance curves for IV injected oxytocin generated using both raw (red) and extracted (blue) plasma, with confidence intervals (dashed).

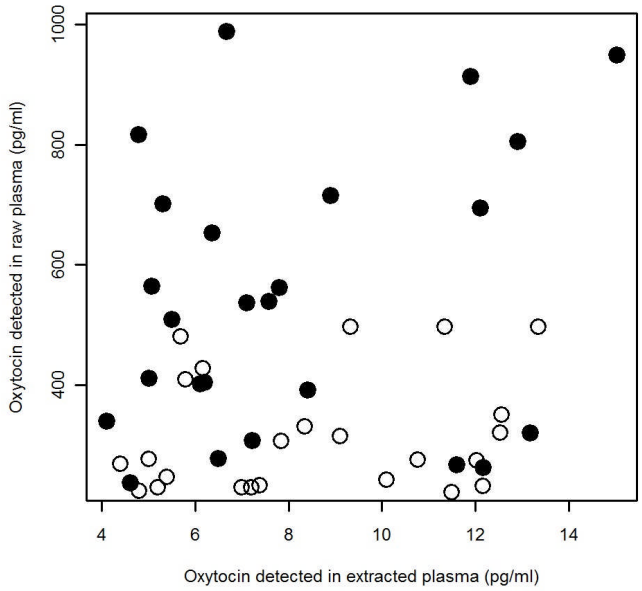


Figure 3-4. Oxytocin concentrations detected in plasma samples that were analysed both raw and with extraction prior to assay. Samples collected in EDTA vacutainers are shown in black (●) and samples collected in heparin vacutainers are shown in white (○).

3.3.4. The effect of capture and handling stress on plasma oxytocin concentrations

No relationship was found between oxytocin concentration detected in extracted plasma and the use of chemical or physical restraint in the capture process (n=36 GLM, $F_{1, 35} = 0.73$, p=0.27), time taken to obtain a sample from first contact (n=36 GLM, $F_{1, 35} = 1.03$, p=0.14), the interaction term for time to sample and type of restraint (n=36 GLM, $F_{1, 35} = 2.41$, p=0.13) or vacutainer type (n=36 GLM, $F_{1, 35} = 0.61$, p=0.39) (Table 3-3 and Figure 3-5).

There were significant differences in detected oxytocin concentrations in raw plasma with the time taken to obtain a sample from first contact (n=36 GLM, $F_{1, 35} = 0.62$, p=0.007), the interaction term for time to sample and type of restraint (physical or chemical) (n=36 GLM, $F_{1, 35} = 0.73$, p=0.01) and vacutainer type (n=36 GLM, $F_{1, 35} = 28.8$, p<0.001) (Table 3-4 and figure 3-6).

Table 3-3. Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in extracted plasma, their estimates, standard errors and p values.

GLM variable	Estimate	Standard Error	p-value
Time to obtain sample	0.28	0.18	0.14
Type of restraint	0.34	0.31	0.27
Vacutainer type	0.09	0.11	0.39
Interaction term between time to sample and type of restraint	-0.29	0.18	0.13

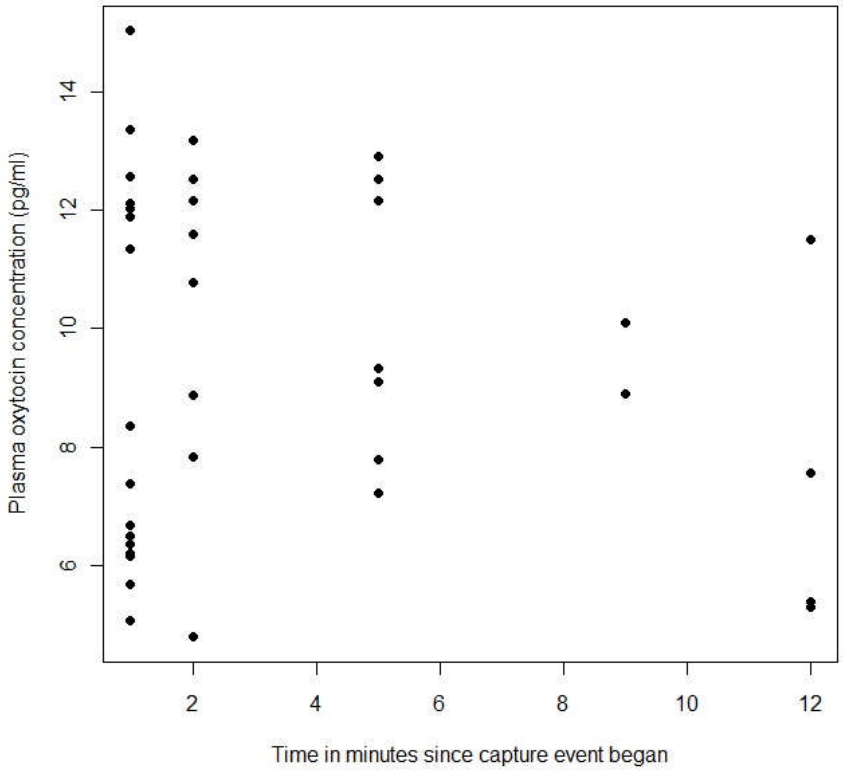


Figure 3-5. Relationship between plasma oxytocin concentration and time spent restrained prior to blood sampling using extracted samples.

Table 3-4. Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in raw plasma, their estimates, standard errors and p values.

GLM variable	Estimate	Standard Error	p-value
Time to obtain sample	-0.53	0.18	0.007
Type of restraint (Chemical)	-0.39	0.31	0.21
Vacutainer type (Heparin)	-0.57	0.11	<0.001
Interaction term between time to sample and type of restraint	0.5	0.18	0.01

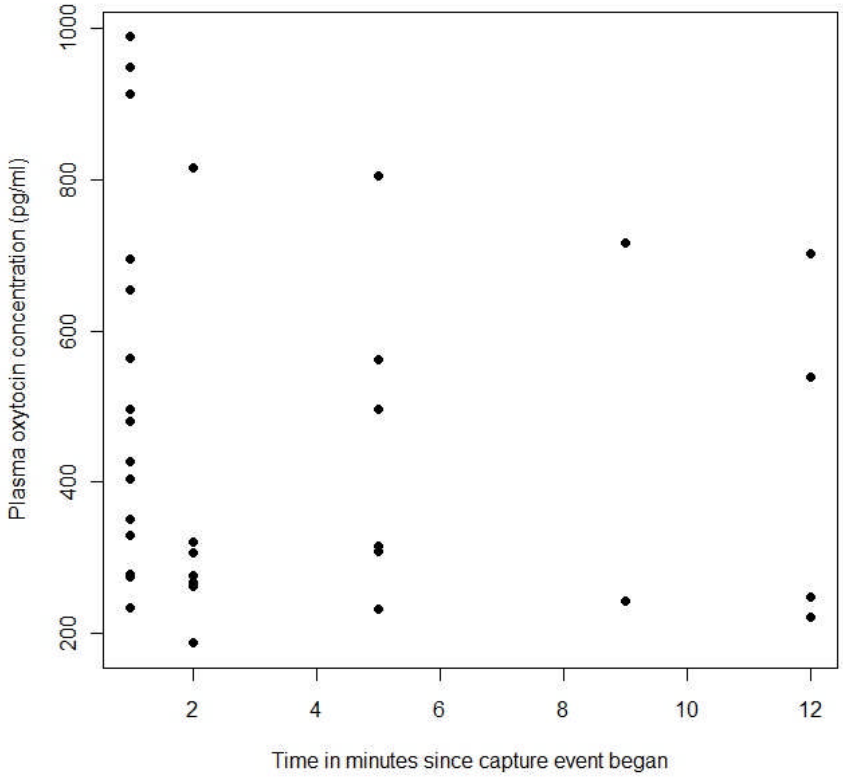


Figure 3-6. Relationship between plasma oxytocin concentration and time spent restrained prior to blood sampling using raw plasma samples.

3.3.5. Protocol for the detection of oxytocin concentrations in phocid milk.

Of the four mothers that had six point serial milk dilutions run raw on the ELISA plate, all contained oxytocin concentrations too high to be detected on the detection curve generated in the Assay Designs ELISA. Samples that were extracted from 3ml and 1.5ml of milk prior to analysis were detectable on the plate however. The mean milk oxytocin concentration from these two grey seal mothers was 147.1pg/ml (SD: 61.3).

3.4. Discussion

3.4.1. Assay selection for the detection of plasma oxytocin concentrations in phocid seals.

All assays tested were able to detect oxytocin in seal plasma to some degree. Concentrations in raw plasma and extracted plasma were similar to published data on oxytocin levels in other species (Martinez-Lorenzana *et al.* 2008 and Cool and DeBrosse 2003). However, the RIA did not detect as many samples of extracted plasma as the ELISA, suggesting that ELISAs are more sensitive and the better choice of assay for the purposes of this project. This, coupled with advice from leaders in this field of behavioural endocrinology (C.S. Carter, pers. comm), lead us to rule out RIA as part of our protocol. While the Phoenix Pharmaceuticals ELISA did detect more samples than the RIA, the extracted results were skewed towards the left side of the standard curve, making the results more susceptible to errors caused by small changes in the absorbance reading. In subsequent kit tests we therefore adapted the protocol to ensure that more samples were detected towards the middle of the curve, to allow for more accurate calculation of results. To achieve this, the volume of plasma used to extract from was increased from 1ml to 3ml for the Assay Designs ELISA. All samples were detected on the plate, but nine were too high to quantify on the standard curve leading to another trial of this plate using oxytocin extracted from 1.5ml of plasma. With this protocol, all samples run on the Assay designs plate were detected. The excellent detection rates, coupled with the cheaper cost of the Assay Designs kit (£100 less per kit than Phoenix Pharmaceuticals), lead to the selection of this ELISA for all future plates used for the analysis of samples for this PhD thesis.

3.4.2. Effects of sampling and analysis protocol variables on plasma oxytocin concentrations.

This study found clear and substantial differences in the oxytocin concentrations measured in phocid plasma samples depending on the protocol used to collect and analyse them. The largest differences were between raw and extracted plasma: raw samples produced oxytocin measures several hundred times higher than extracted plasma. Samples taken with EDTA vacutainer tubes had significantly higher

concentrations of oxytocin than heparin tubes when run raw on a plate, but when extracted there was no difference between the two. Therefore the design of a study protocol will have a major impact on the results generated, which must be taken into account when analysing and interpreting the data.

Our study shows that if plasma is extracted prior to analysis, the particular anticoagulant in the vacutainer makes no difference to the oxytocin detected. This allows studies using extracted plasma to be comparable, regardless of vacutainer choice. However, when analysing raw plasma, EDTA tubes contain almost double the concentration of oxytocin compared to heparin tubes collected samples (mean oxytocin EDTA: 543.2pg/ml, heparin: 300.9pg/ml). These concentrations represent the first published data on plasma oxytocin concentrations found in a phocid seal, or any marine mammal species, and are comparable to both extracted and raw plasma concentrations reported in the literature for other mammalian species. Humans have been reported to have basal plasma oxytocin concentrations ranging between 0.1-23pg/ml in extracted plasma and 99-405pg/ml in raw plasma (reviewed in Szeto *et al.* 2011) and rats have mean oxytocin concentrations of 6.8pg/ml in extracted plasma (Langraf 1981) and concentrations ranging from 78.9-580pg/ml in raw plasma (Carter *et al.* 2007, Martínez-Lorenzana *et al.* 2008).Other studies analysing different biological compounds have reported similar differences between the anticoagulants (Dong *et al.* 2010 and Gonzalez-Covarrubias *et al.* 2012) while for some peptides there appears to be no difference (Varo *et al.* 2006). The protocol instructions for the Assay Designs ELISA does state that the chelators in EDTA might affect the activity of the conjugate used in the kit (Enzo Life Sciences Oxytocin ELISA kit Manual 2012). The conjugate is responsible for reacting with the p-nitrophenylphosphate substrate to generate a yellow colour, which indicates a low concentration of oxytocin as the intensity of the colour increases. If it was not functioning properly due to EDTA presence, then no yellow colour would be generated and a false positive would occur. Heparin vacutainers work differently by potentiating the action of antithrombin III (Chuang *et al*. 2001), which inactivates coagulation proteins rather than using chelators to bind ions, and may account for the difference in results. This highlights the importance of fully investigating the consequences of any deviation from the kit manufacturer's instructions as the kit protocol recommends using EDTA tubes for collection with extracting prior to analysis. Therefore the discrepancy between the two tube types must be taken into account when designing a study, interpreting the

results and comparing them across studies, and can be avoided completely if samples are extracted.

The individual's species and sex were both significant sources of variation in this study, while time of year was not. As this work includes few individuals, and no repeats of any species/sex combination were possible, stochastic differences between them are entirely plausible, and having access to a larger number of animals would have allowed us to examine this variation to determine its validity. Despite there being significant variation in individual's oxytocin concentration, the differences generated by the four protocols are greater still and are present at the highest level of significance in the model.

Finally this study focused on the variation in oxytocin generated by extraction protocol and vacutainer choice, and did not specifically test for variation generated by differing storage temperatures or the use of aprotinin to stabilise the peptide. These two variables were kept consistent across the samples in this study, with all plasma stored at -20ºC with no addition of aprotinin. The coefficient of variance in this study remained consistently below 5% across two years of analysis, indicating that degradation of oxytocin in samples stored at -20ºC without the use of aprotinin was negligible. The influence of storage temperature and additional inhibitors on oxytocin concentration detected would be an area worthy of future study, as protocols in the literature vary on these points along with vacutainer type and use of extraction. The majority of studies published freeze at the recommended -70ºC with a minority using -20ºC or below (Scantamburlo *et al.* 2007, Yayou *et al.* 2010), yet this study and several others (Szeto *et al.* 2011, van der Post *et al.* 1997) indicate that oxytocin may be more stable in warmer storage temperatures than is thought currently. The use of aprotinin or any inhibitor post-sampling is extremely varied in the literature, with as many studies using aprotinin (Feldman *et al.* 2010a and 2010b, Grewen *et al.* 2010, Taylor *et al.* 2010, Skrundz *et al.* 2011, Hoffman *et al.* 2012, Weisman *et al.* 2013a) as not (Scantamburlo *et al.* 2007; Yayou *et al.* 2010; Szeto *et al.* 2011; Deisenhammer *et al.* 2012; Hoge *et al.* 2012, Gossen *et al.* 2012). The impact of these additional inconsistencies between studies in oxytocin analysis protocol would benefit from further investigation.

From these results, we concluded that all samples collected for this project should be extracted prior to analysis, and that the type of anticoagulant used in the vacutainers would not matter as long as the sample underwent extraction. Finally, -20ºC freezer

temperatures without the use of aprotinin create suitable conditions to preserve oxytocin in samples taken for this project, which will allow us to utilise samples from remote field sites where freezing temperatures of -70ºC would not be feasible.

3.4.3. Relationship between extracted and raw oxytocin plasma concentrations

Our results show clearly that the concentration of oxytocin detected in raw plasma samples is hundreds of times higher than that in extracted samples (mean oxytocin extracted EDTA: 8.1pg/ml, extracted heparin: 8.3pg/ml, raw EDTA: 543.2pg/ml, raw heparin: 300.9pg/ml). Szeto *et al.* (2011) came to the same conclusion in their paper evaluating radioimmunoassay and ELISA protocols for oxytocin analysis. They used high performance liquid phase chromatography (HPLC) to investigate the components of raw plasma, enabling them to identify several oxytocin immunoreactive species in raw plasma. These account for the large difference between the raw and extracted samples, and at least one of these was identified as a degradation product of oxytocin. It is vital that any studies using raw plasma as their medium for analysis take this into account, as any patterns that may appear in such samples will not be a reflection of oxytocin alone, but of an unknown number of other metabolic processes generating these reactive molecules in the blood.

The potential differences a study can incur in their results just by using raw or extracted plasma is well illustrated by the different plasma clearance rates generated from the clearance trial in this study. The plasma clearance rate for seals detected with extracted plasma (0.035L/min/kg) is comparable to the existing values published for the clearance rate of oxytocin in other mammalian species detected with RIAs (0.027 L/kg/min in human men and 0.021L/min/kg in human women (Leake *et al.* 1980), between 0.025 and 0.085L/min/kg in rats depending on dose (Morin *et al.* 2008), and 0.016L/min/kg in baboons (Kowalski *et al.* 1998)). However the plasma clearance rate generated by the raw plasma data (0.004L/min/kg) is much slower than the rate from extracted samples and other published work due to the high AUC value generated from the raw plasma clearance curve.

A difference between the two concentrations detected does not immediately rule out that information about one cannot be inferred from the other. If the other reactive species measured in the ELISA in raw samples are products of oxytocin metabolism then it could be theorised that raw levels would still give an indication of an

individual's oxytocin activity in the blood. There have been studies that detected such a correlation (Michopoulos *et al.* 2011) and others that found no such link (Szeto *et al.* 2011). This study was not able to find any correlation between the raw and extracted samples, and in addition found that regression models built on such data had poor predictive power for generating accurate extracted oxytocin levels from raw measurements. When analysing a set of serial samples taken during a clearance trial, the data generated from extracted samples produced a curve which accurately represented the decay rate. The data generated from raw samples, while still detecting an overall fall in oxytocin levels, could not have a clearance curve fitted to it that accounted for more than 30% of the variation in the data. Therefore our findings are in agreement with Szeto *et al.*'s work, that raw plasma cannot be used to accurately analyse oxytocin levels. However, Michopoulos *et al*. used serum rather than plasma for their analysis, which may have produced differences in the metabolites maintained or denatured during collection and storage. In addition the datasets used here and by Szeto *et al.* to generate the regressions and correlations are larger (n=52 and n=39 respectively) than the one used by Michopoulos *et al.* (n=11) and therefore may have obtained a more representative dataset to work from. Michopoulos *et al.* also do not give details of the correlation model used to generate their results, preventing direct comparisons between the varying methods of statistical analysis used here (GLM) and by Szeto et al. (Spearman Rank Correlation).

Therefore we caution that studies using raw plasma with the Assay Designs ELISA kit are unlikely to report oxytocin alone and additionally cannot accurately infer this data from their results. These results further reinforced our decision to extract all samples prior to analysis, to gain the most accurate and repeatable results possible from our samples.

3.4.4. The effect of capture and handling stress on plasma oxytocin concentrations

The stress from being manually or chemically restrained for the blood sampling event or the time taken to obtain a sample had no effect on plasma oxytocin concentrations providing the samples were extracted prior to analysis. Oxytocin has been linked to restraint stress in rodents (Grippo *et al.* 2009) and is hypothesised to rise in response to high cortisol levels. However in previous studies only 'extreme' long term capture

and restraint protocols generated peaks in oxytocin (C.S. Carter 2010, pers. comm) and our results support this. As there were no significant changes in oxytocin concentration with time, it should be possible to obtain samples that allow detection of basal oxytocin concentrations from wild animals captured either manually or using a chemical immobiliser.

3.4.5. Protocol for the detection of oxytocin concentrations in phocid milk.

The concentration of oxytocin in phocid milk samples was successfully detected in two individuals. Currently only two other studies have published milk oxytocin concentrations in humans (Leake *et al.* 1981) and cows (*Bos primigenius)* (Prakash *et al.* 2009), and both detected concentrations much lower than those found in grey seals (10pg/ml of oxytocin in the milk of their respective study species). The high oxytocin concentrations detected in phocid milk compared to cows and humans is difficult to account for given the poor understanding of oxytocin's function in milk. It has been hypothesised that ingestion of milk is an important source of neonatal exposure to oxytocin (Uvnäs-Moberg 1998, Carter 2003), which has been shown to have life long developmental consequences for the infant in rodents (Sohlstrom *et al.* 2000, Olausson *et al.* 2003, Bales *et al.* 2004, Cushing *et al.* 2005, Bales *et al.* 2007). Infant cattle and humans are dependant on their mothers for far longer than seal pups, with nursing occurring beyond 6 months in both cattle (Lidfors *et al.*1994) and humans (Reinhardt and Reinhardt 1981). The higher oxytocin concentrations in grey seal milk may reflect the short lactation period found in this species. Neonatal oxytocin exposure has additionally been found to negate the negative consequences in the infant of maternal malnutrition in rats (Olausson *et al.* 2003) which could be important in phocids. Grey seal mothers must fast during lactation, and loose approximately a third of their body mass while raising a pup (Iverson *et al.* 1993). Neither cattle nor human mothers fast while nursing dependant offspring, and therefore they may not require high oxytocin concentrations to be given to the infant to prevent disruption of its development due to malnutrition. However, oxytocin was only successfully detected in the milk of two individuals in this study, and larger samples size should be analysed to ensure that a representative dataset has been generated on the oxytocin concentrations in this substrate for this species.

3.5. Conclusions

In conclusion, the protocol followed when collecting and analysing plasma for oxytocin does have a significant impact on the results generated and experimental design should be carefully considered when planning research in this area. Presence or absence of an extraction stage in the analysis protocol generates the largest amount of variation in the oxytocin concentrations detected; therefore the protocol including extraction should be used for all samples. The Assay designs ELISA plate is sensitive enough to detect oxytocin in extracted samples from both mother and pup grey seals from low volumes of plasma (1.5ml), allowing maximum detection and extraction efficiency. The concentrations of basal plasma oxytocin in phocid seals are consistent with those detected in all mammal species studied to date in both extracted and raw plasma, and the clearance rate for this hormone in phocids is also comparable to that found in humans, rats and primates. Therefore despite being novel species to this field, it appears that phocid seals represent a typical mammalian system in terms of plasma oxytocin. Finally the capture and handling techniques needed to obtain plasma samples from seals does not introduce any spikes or losses of oxytocin, allowing accurate detection of basal concentrations and peaks generated by natural stimuli, which is crucial for the success of the studies in this thesis.

The findings presented in this chapter have been accepted for publication in the Journal of Neuroscience Methods and is currently in press. Please see Appendix D for a copy of this paper.

Chapter 4: Quantifying the Maternal Behaviour of the Grey Seal

4.1. Introduction

Mammalian maternal behaviour typically requires a substantial investment of time and energy to be successful (Trillmich 2010). For each species, sub-population and individual there are optimal strategies and patterns of behavioural expression that maximise reproductive success. However, within populations, there are often observations of individuals failing to successfully rear offspring, or rearing offspring inefficiently in terms of energetic investment (Perry *et al.* 1998). Within species, the maternal behaviour an individual displays can be quite varied (Fairbanks 1996), with a range of factors influencing its expression and the level of maternal investment in each reproductive episode. These include age of the individual (Clutton-Brock 1984), the habitat they occupy (Conradt *et al.* 1999), the nutritional constraints on the mother during the dependant period (DeGabriel *et al.* 2009) or the physiology underlying the control of the behaviour (Maestripieri *et al.* 2009). Before the underlying causes of such variation can be investigated, or what cost specific behaviours may incur to the mother or offspring, a baseline of the typical variation in the maternal behaviour of a species or population must be established. If there are behaviours that are performed persistently at cost to the mother or infant, it can be considered to be maladaptive. Its presence in the behavioural repertoire of that individual or species therefore becomes difficult to understand, as in theory it should be selected against in a population (Darwin 1859).

Many studies document grey seal reproductive and maternal behaviour and these offer a good starting point for this thesis. There can be wide variation in the success grey seal mothers have in exhibiting maternal care and raising a pup to weaning (Boyd and Campbell 1971, Fogden 1971, Anderson *et al.* 1975, Kovacs 1987), especially between different breeding colonies (Caudron 1998, Perry *et al.* 1998). Previous behavioural studies examine a variety of aspects of maternal behaviour such as perinatal activity (Fogden 1971, Burton *et al*. 1975), effects of topography (Anderson and Harwood 1985, Twiss *et al.* 2000, Redman *et al.* 2001), reproductive energetics (Haller *et al.* 1996, Pomeroy *et al.*1999), methods of mother-pup recognition

(McCulloch *et al.* 1999, McCulloch and Boness 2000) and causes of pup mortality (Anderson *et al.* 1979, Baker 1984, Baker and Baker 1988, Twiss *et al*. 2003). Previous studies quantifying behavioural data from grey seals highlight particular behaviours that may be important indicators of maternal quality or type, such as the frequency of 'pup checking' (Twiss *et al.* 2012a) or 'alert' behaviours (Culloch 2012). However the protocols for collecting such data are often tailored to the individual needs of each study based on the questions identified in the study and logistical limitations of study sites, and this may affect the value of comparisons across multiple datasets. Some behavioural data are collected around rare, infrequent or difficult to observe but important behaviours such as aggressive conflicts (Redman 2002), births (Burton *et al.* 1975) and mother-pup separations and reunions (Redman *et al.* 2001). Other studies use timed scanning of multiple individuals spread throughout a colony to gather data on the frequency that individuals exhibit different behaviours throughout the day (Anderson *et al.* 1985, Twiss *et al.* 2000, Twiss *et al.* 2002, Culloch 2012). Such differences in data collection methodologies are important to keep in mind when designing a study and in comparing findings across existing ones to interpret results. Therefore it is essential to collect data specifically for use in this thesis to generate data exactly suited to the questions and analyses of later chapters. Maternal behaviours that have the potential to impact negatively on the mother or pup in grey seals have been described, such as abandonment (Fogden 1971), fostering (Perry *et al.* 1998), allosuckling (Boyd 1971, Baker 1984, McCulloch et al. 1999) and attempts to nurture after death (Kovacs 1987, Twiss *et al.* 2003, K.J. Robinson, pers. obs). Such accounts are often opportunistic, providing only a record that the behaviour does exist in this species. Phocid seals show great variety in the expression of these behaviours, for example the occurrence of allosuckling in Weddell seals is 'low' (Tedman and Bryden 1979) while in Mediterranean monk seals (*Monachus monachus*) approximately 50% of observed females exhibit this behaviour (Cedenilla *et al* 2009). Only one study to date contains quantification of the natural frequency of fostering behaviour in breeding grey seal females: Perry *et al.* (1998) documented the occurrence of fostering behaviour across three grey seal colonies in the northern hemisphere. They found that different colonies had different frequencies of fostering behaviour, ranging from 2% to 28% of observed females adopting non filial pups. The North Rona site was not included in this study; therefore nothing is know about the prevalence of this behaviour on this colony.

This chapter documents the range of maternal and pup behaviour in wild, free ranging grey seal mother-pup pairs on the breeding colony of North Rona. The basic description of maternal behaviour provided in this chapter will provide the essential baseline data for examining the role of oxytocin in maternal behaviour (Chapter 5).

The objectives of this chapter were to:

1. Investigate the impact of data collection method (scanning frequency) on the behavioural profiles recorded in order to establish the most suitable observational protocols to quantify maternal behaviours.
2. Collect a large direct observation database of maternal behaviour for different individuals and breeding years to allow detection of the potential range of behavioural expression, and analyse data for critical components of variance to allow further analysis with endocrinological variables.

4.2. Methods

4.2.1. Study sites and animals

Female grey seals with dependent pups were observed on North Rona during the 2009, 2010 and 2011 breeding seasons from early October until early November. For more details on the North Rona study site, please refer to Chapter 2. Individuals observed were all females which had previously been photographed and given an identity in the long term dataset of returning breeding females for this colony. These individuals were identified by their pelage patterns, tags or the presence of brand markings. Females were identified in the colony as soon as possible, preferably before pupping, and observations did not begin until the females had pupped. If identification was made after the pup was born, only mothers with young (stage 1, Kovacs 1987) pups were included in the study. In each of the study years there were 28 or more mother-pup pairs observed (Table 4-1), with a subset of these used for detailed scan data collection depending on their visibility from the hide location. Eleven females were recorded on the colony in all three years of the study, and eight were recorded in two years.

Table 4-1. Numbers of observed mother/pup pairs on North Rona from 2009 – 2011

Year	Number of mother/pup pairs observed	Number of mother/pup pairs with detailed scans
2009	35	18
2010	38	15
2011	28	13

4.2.2. Behavioural observations

The ethogram described in Chapter 2. was used for all observational data collected across all three years. Observations were conducted using two different scanning methodologies over the three years of the study. In 2009 data were collected from October 6th until October 31st (total: 104 hours). Scans of the study group were made every five minutes on between six and fifteen mother/pup pairs depending on how many study females were present and identifiable on the colony each day. This scanning protocol was used in the first study year of the project as it was closely based on an existing protocol for grey seal behavioural data collection and permitted collection of data from as many mothers as possible. The scanning protocol was subsequently changed to allow greater detail of the behaviour of fewer individuals to be collected in 2010 and 2011 to maximise the likelihood of documenting infrequent yet crucial maternal behaviours such as suckling bouts. Data were collected between September 28th – November 1st 2010 (total: 220 hours) and September 30th – October 29th 2011 (total: 153 hours). Two mother-pup pairs were observed over nine consecutive hours every day using scans every 30 seconds for 15 minutes, followed by a five minute break to record other data from the colony. The distance from mother to pup estimated visually in adult body lengths was also recorded every 15 minutes (mother-pup distance).

4.2.3. Statistical analysis

Behavioural scan data from the mother-pup pairs on North Rona were first converted to percentages of the number of scans attributed to a behavioural category out of the total number of scans for that mother-pup pair per day. Any scans where the mother or pup was recorded 'out of sight' (OOS) were deducted from the total number of scans for that day.

To collapse the ethogram described in Chapter 2 into fewer, broader behavioural groupings, biologically plausible combinations of ethogram categories were considered. Of the 35 ethogram categories, one was not included in subsequent analysis (comfort move (CM)) and twelve were infrequently recorded (recorded in <3 days of the total month long study period) and were therefore eliminated from subsequent analysis (general behaviours: 'other', chase, flee and vocalisation, mother behaviours: birth, attempted copulation, failed copulation and copulation, pup behaviours: thrown, bitten, dead and eaten). The remaining 22 categories were then combined into seven broader groups, as described in Table 4-2. Principle component analysis (PCA), which identifies the behaviours that account for the majority of the variance in the data, was then used to determine if any of the seven behavioural groupings could be combined for future analysis. PCA analysis was conducted on data collected in 2010 and 2011 for mother-pup pairs which had corresponding blood samples collected to allow analysis with oxytocin concentrations in Chapter 5. PCA analysis was conducted on the seven groupings with orthogonal rotation (varimax) using the procedures for personality and psychological research library 'psych' (Revelle 2013). Bartlett's test of sphericity indicated that correlations between groups for both the mother, $\chi^2_{21}=261.6$, $p<0.001$, and pup, $\chi^2_{21}=272.7$, $p<0.001$ data were sufficiently large for PCA.

Three two way ANOVAs were used to determine whether scanning regime produced different estimates of the percentage time an individual spent in three different behaviours, while additionally controlling for individual mother identity. Based on the results of the PCA analysis, the percentage time spent performing an important but short duration behaviour ('check pup', CKP), a frequent behaviour (resting, RES) and an important infrequent behaviour (nursing, SUC) was compared across the two sampling regimes used in the three years of the study. The effects of different scan sampling regimes were assessed using data from three females with similar durations

of observations during similar stages of pup development across all three study years (2009, 2010 and 2011).

Table 4-2. The seven behaviour groupings used for the PCA and which ethogram categories (from Chapter 2) were included in them.

Mother/Pup	Behaviour group	Ethogram Categories Included
Mother	Resting	Resting (RES) and head up rest (HUP)
	Nursing	Presenting to Pup (PRE) and nursing (SUC)
	Interacting with pup	Interacting with Pup (INP) and flippering (FLP)
	Checking pup	Alert to pup's presence or activity, excluding the surroundings (CKP)
	Alert	Alert to surroundings, not including pup (ALE)
	Aggression	Open Mouth Threats to males and females (OMTM and OMTF), rapid sequences of aggression (SCRAP), aggressive flippering (FLPAG), lunges (LUN) and bites (BIT)
	Locomotion	Locomotive movement for any purpose (LOC)
Pup	Resting	Resting (RES)
	Suckling	Suckling (SUC) and potentially suckling (SUC?)
	Interacting with mother	Interacting with mother (INM) and nosing mother (PNOS)
	Checking mother	Alert to mother's presence or activity, excluding the surroundings (CKM)
	Alert	Alert to surroundings, not including mother (ALE)
	Play	Exploration of surrounding soil, rocks and plants (EXP) and play (PLY)
	Locomotion	Locomotive movement for any purpose (LOC)

4.3. Results

4.3.1. Impact of scanning protocol on estimations of behaviour frequencies

Neither of the two variables tested (protocol or mother identity) had a significant effect on the percentage time spent in the three behaviours tested. Protocol did not impact on the percentage of time a mother spent checking her pup ($F_{2,17}$=1.6, p=0.24), suckling her pup ($F_{2,17}$=1.4, p=0.29) or resting ($F_{2,17}$=0.46, p=0.64) (Table 4-3). The identity of the mother did not significantly affect the percentage time spent in any of the three behaviours tested (checking pup; $F_{2,17}$=0.23, p=0.79, suckling; $F_{2,17}$=2.89, p=0.09, resting; $F_{2,17}$=0.44, p=0.65).

Table 4-3. Mean values with standard errors for the percentage time spent resting, pup checking and suckling from three grey seal mothers on North Rona across three different years collected with two different sampling methodologies (the same sampling protocol was used in both 2010 and 2011).

Behaviour	**2009**	**2010**	**2011**
Resting	82.97 (±2.49)	79.55 (±2.37)	78.28 (±4.87)
Checking pup	2.19 (±0.74)	3.72 (±0.25)	3.33 (±0.67)
Suckling	6.07 (±1.07)	4.86 (±0.84)	3.49 (±1.64)

4.3.2. Maternal behaviour of grey seals on North Rona

Of the seven behaviour groupings defined in the methods section of this chapter, four components were required to account for at least 80% of the variation in the data for both mother and pup behaviours (Tables 4-4 and 4-5 and Figures 4-1 and 4-2). Tables 4-6 and 4-7 show the component loadings after rotation for both mother and pup PCA. Proportion of time spent resting was consistently negatively correlated to other behavioural categories in all components detected in the PCA in both mother and pup datasets. Therefore it was analysed as a separate component in subsequent chapters (Chapter 5), giving a total of five components of behaviour. Summaries of the mean percentage time spent in the five components combined by the PCA analysis are shown in Table 4-8.

Table 4-4. The five components accounting for 80% of the variation in the behavioural data exhibited by mothers on North Rona in 2010 and 2011 and the behavioural groupings they include from Table 4-2.

Component name	**Behavioural groupings included**
Awareness of pup	Checking pup and locomotion
Pup care	Nursing and interacting with pup
Aggression	Aggression
Alertness	Alert
Maternal resting	Resting

Table 4-5. The five components accounting for 80% of the variation in the behavioural data exhibited by dependant pups on North Rona in 2010 and 2011 and the behavioural groupings they include from Table 4-2.

Component name	**Behavioural groupings included**
Interaction with mother	Suckling and interacting with mother
Pup activity	Locomotion and Alert
Play behaviour	Play
Awareness of mother	Checking mother
Pup resting	Resting

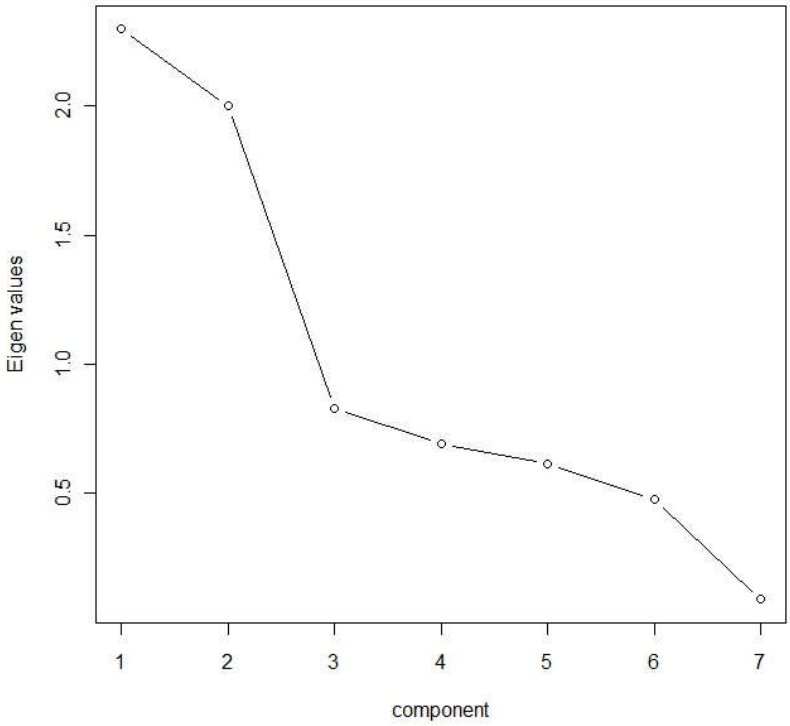


Figure 4-1. Scree plot of the PCA for mother behavioural data.

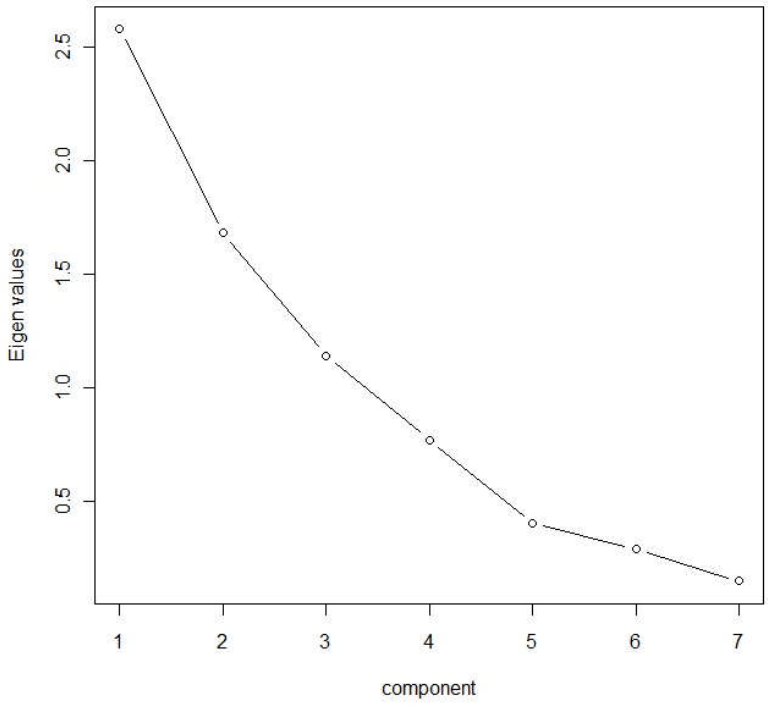


Figure 4-2. Scree plot of the PCA for pup behavioural data.

Table 4-6. The component loadings for the PCA on the seven categories of mother behavioural data, with the loadings for the behaviours included in each component in bold.

Behavioural Group	**PCA components**			
	Awareness of pup	Pup care	Aggression	Alertness
Resting	-0.49	-0.79	-0.28	0.01
Nursing	-0.24	**0.9**	-0.1	-0.12
Interacting with pup	0.19	**0.49**	0.02	-0.73
Checking pup	**0.75**	0.16	0.28	-0.11
Alert	0.43	0.16	0.27	**0.73**
Aggression	0.11	0.01	**0.96**	0.12
Locomotion	**0.74**	-0.23	-0.1	0.34

Table 4-7. The component loadings for the PCA on the seven categories of pup behavioural data, with the loadings for the behaviours included in each component in bold.

Behavioural Group	**PCA components**			
	Interaction with mother	Pup activity	Play behaviour	Awareness of mother
Resting	-0.47	-0.34	-0.72	-0.23
Locomotion	0.13	**0.91**	0.04	0.14
Playing	-0.07	0.00	**0.97**	0.05
Checking mother	0.15	0.13	0.13	**0.96**
Alert	-0.16	**0.9**	0.11	0.02
Suckling	**0.91**	-0.04	0.07	-0.03
Interacting with mother	**0.84**	0.01	0.01	0.25

Table 4-8. Mean values with standard deviations for the percentage time spent by mothers and pups in the five different PCA components detected (rest, awareness of pup, pup care, aggression and alertness for mothers and rest, interaction with mother, pup activity, play behaviour and awareness of mother for pups).

	Mothers		**Pups**	
Component	**Mean %**	**SD**	**Mean %**	**SD**
Rest	81.07	4.81	66.73	9.94
Awareness of pup	4.94	1.56	na	na
Pup care	6.25	3.33	na	na
Aggression	0.76	0.69	na	na
Alertness	3.62	1.29	na	na
Interaction with mother	na	na	6.32	6.19
Pup activity	na	na	5.81	3.77
Play behaviour	na	na	17.37	5.15
Awareness of mother	na	na	1.37	0.93

4.4. Discussion

4.4.1. Impact of scanning protocol on behaviour data recorded

The frequency of scanning each mother during an observation period made no difference to this study's ability to detect common, infrequent or brief behaviours for that individual across years. This allows for all three years of behavioural data to be utilised in this thesis without concerns that variability is being introduced to the data due to different methodology. However, viable plasma samples for endocrinologcial analysis were only collected in 2010 and 2011; therefore no 2009 behavioural data were used in any subsequent analysis in this thesis.

4.4.2. Maternal behaviour in grey seals on North Rona

The analysis presented in this chapter provides five groupings of the behavioural categories that document the mother and pup behaviour recorded on the North Rona colony in 2010 and 2011. These five components account for the largest amount of

variance in the data while collating it into manageable categories for further analysis with endocrinological data in Chapter 5. For the majority of their time on a colony (81.1%, SD = 4.81), mothers with dependent pups rest, conserving energy by staying in one place and reducing the chance of separation from their pups. This is reflected in the low mean mother-pup distance of 1.3 body lengths (SD=1.5). This figure for percentage time spent resting is comparable to that found by other studies on maternal grey seal time budgets (Table 4-10), as is the time spent in all other components. The importance of the Checking pup and Alert behaviour has been highlighted in previous studies as being a key characteristic that allowed individual mothers and their maternal strategies to be differentiated (Twiss *et al.* 2012a and 2012b, Culloch 2012). Our data supports these findings as an entire component of the variation in the data is due to the Checking pup behaviour combined with the amount of locomotion a mother performs, and a separate one is solely caused by Alert behaviours.

Table 4-9. Summary of reported figures for the percentage time spent in different behaviours by grey seal pups on a breeding colony. Behaviour categories are as defined in Table 4-2, ethograms of the cited studies were checked for consistency with the ethogram in Chapter 2, where appropriate categories from published studies were combined to allow comparison with the categories in Table 4-2.

Study	Haller *et al.* 1996	Twiss *et al.* 2000	Twiss *et al.* 2000	Twiss *et al.* 2012b
Mean/median	Mean	Median	Median	Mean
Study Site	Amet Island, Canada	West Rona beach, North Rona, Scotland	Tarbet, North Rona, Scotland	North Rona, Scotland
Behaviour (%)				
Resting	73.3*	67.16	66.13	na[1]
Suckling	5.83*	2.11	2.7	na[1]
Alert	2.3*	na[2]	na[2]	na[2]
Locomotion and Play	9.3*	29.69	30.51	19.51

na[1] Not recorded in this study
na[2] Included in the 'Locomotion and Play' category
*Percentages presented here are the means of pups born in early, mid and late season

Table 4-10. Summary of reported figures for the percentage time spent in different behaviours by grey seal mothers on a breeding colony Behaviour categories are as defined in Table 4-2, ethograms of the cited studies were checked for consistency with the ethogram for this thesis (Chapter 2), where appropriate categories from published studies were combined to allow comparison with the categories in Table 4-2.

Study	Anderson and Harwood 1985	Anderson and Harwood 1985	Haller *et al.* 1996	Haller *et al.* 1996	Twiss *et al.* 2000	Twiss *et al.* 2000	Twiss *et al.* 2012b	Culloch 2012
Mean/median	Mean	Mean	Mean	Mean	Median	Median	Mean	Median
Study Site	North Rona, Scotland	Monarch Isles, Scotland	Amet Island, Canada	Gulf of St Lawrence, Canada	West Rona beach, North Rona, Scotland	Tarbet, North Rona, Scotland	North Rona, Scotland	North Rona, Scotland
Behaviour (%)								
Resting	79.7	71.0	86.2	89.5	79.09	77.11	79.09	79.76
Nursing	1.8	3.6	8.4	6.1	na[1]	na[1]	10.15	3.79
Interacting with pup	1.2	1.1	0.7	0.4	5.19	4.55	1.71	1.28
Checking pup	na[2]	na[2]	3.0	2.2	na[2]	na[2]	2.46	1.97
Alert	10.2	15.0	11.2	13.6	10.42	11.43	6.54	6.04
Aggression	1.0	1.4	2.0	1.4	1.72	1.79	0.74	0.72
Locomotion	1.3	4.6	2.4	1.0	0	1.96	1.43	0.65

na[1] Nursing behaviours included in the Interacting with pup behaviours. na[2] Checking pup behaviour included in the Alert behaviour.

Pups spend the majority of their time on a colony resting (66.7% SD=9.94) or in behaviours such as playing and exploring their surroundings (17.4% SD=5.15). The figures for the pup time budgets are more difficult to compare to existing studies due to more differences in ethograms existing across studies (Table 4-9), however the figures for the time pups spend resting are similar in that in all studies and colonies studied, pups spend less time resting than their mothers (Haller *et al.* 1996, Twiss *et al.* 2000 and 2012b). Resting and playing behaviours typically involve the pup remaining in one place or only moving a small distance over a long time period and are important for two reasons: Firstly these behaviours keep the pup relatively stationary and, therefore, in close proximity to the mother for a large proportion of the time. Even if the mother does move to a different location on the colony, such as when bathing in pools (Redman *et al.* 2001, Stewart *et al*. in press), when she returns the pup will still be in the same region that she left it. Such a high frequency of these 'idle' behaviours may therefore contribute to lowering the number of mum-pup separations that occur during a season, which ultimately prevents a pup dying from starvation on a colony, which is the largest cause of death for grey seal pups in the UK (Boyd and Campbell 1971, Anderson *et al.* 1979, Baker and Baker 1988). Second, large proportions of time in idle behaviours may be important for efficient conversion of milk to blubber and lean body mass. Being inactive reduces the metabolic output of a pup, leading to greater amounts of fat and protein laid down in an individual (Bennett *et al.* 2007). This in turn will positively affect the pup's likelihood of survival in its first year of life (Hall *et al.* 2001 and 2002).

These results show that the majority of behaviour exhibited by mother-pup pairs of grey seals centres around conserving energy and maintaining either a close proximity to each other or a consistent location for the mother to return to. This maximises the efficiency of the energy transfer from mother to pup, and reduces the probability of separation and death for the dependant offspring. The Checking pup and Alert behaviours previously identified in other studies have been found to be a significant source of variation in this dataset also, and the five collated behavioural categories for both mothers and pups allows the behaviours that generate the most variance in the dataset to be simplified and analysed with other variables in future chapters of this thesis.

4.5. Conclusions

This chapter has established a solid dataset describing the typical behaviours expressed by a large set of mother-pup pairs on the North Rona colony. All data collected across the three years of the study could be used in the analysis for this thesis, as scanning frequency makes no difference to our ability to detect different types of behaviour. However, only data that had viable plasma samples taken alongside it were used in subsequent chapters of this thesis. The identification of crucial components to the variance in the data will allow more targeted analysis of any endocrinological factors influencing the expression of maternal behaviour in this species.

Chapter 5: Endogenous Oxytocin and its effects on the Maternal Behaviour of Grey Seals

5.1. Introduction

Maternal behaviour is a crucial aspect of the life history of all mammalian species, as all mammalian mothers must invest energetic resources in bringing one or several foetuses to term and then must continue to invest in their offspring post partum for any reproductive episode to be successful. Selection pressure therefore favours behaviours that minimise costs and risks to the mother while achieving the greatest reproductive output (Trillmich 2010). Despite this, many individuals of mammalian species exhibit maladaptive forms of maternal behaviour, and grey seals are no exception to this. Abandonment (Fogden 1971), fostering (Perry *et al.* 1998), allosuckling (Boyd 1971, Baker 1984, McCulloch et al. 1999), infanticide and pup theft (K.J. Robinson, pers. obs) have all been documented in female grey seals with dependant pups. Conversely extreme expressions of apparent maladaptive 'dedication' in grey seal mothers have also been documented, such as continuing care after the pup has died (Kovacs 1987, Twiss *et al.* 2003), and retrieving pups that have fallen into the sea and risk being swept away from the colony (Boyd and Campbell 1971). Such behaviours are documented in a variety of mammalian species, but the particulars of breeding grey seal physiology and life history provide a unique opportunity to investigate maladaptive behaviour expression in the face of high selection pressure against it:

- Females come ashore to breed annually and do not have other opportunities to give birth in one year (Boness and Bowen 1996).
- Mothers must fast while producing milk with one of the highest fat contents of all mammals (60%, Iverson *et al.* 1993).
- The lactation period and opportunity to invest in their pup is extremely short (18 days, Pomeroy *et al.* 1999).
- The pup's probability of surviving its first year is positively correlated with its mass at weaning (Hall *et al.* 2001, 2002)
- The biggest cause of pup mortality on a colony is starvation due to separation of mothers and pups (Baker and Baker 1988).

- Future reproductive success in the mothers is dependant on the energy expended in current reproductive efforts (Pomeroy *et al.*1999).

Raising a pup to weaning is considered so costly for pinniped species that it has been suggested the most important time for a female to determine whether to continue with this reproductive effort or to abandon it is immediately after birth (Boyd *et al.* 2007). However despite the high energetic cost of nursing a pup, up to 28% of grey seal mothers on a breeding colony have been recorded exhibiting fostering behaviour (Perry *et al.*1998). Why in the face of such costs do some females continue to display behaviours that invest in non-filial pups or practically guarantee the death of their own pup?

Hormones are widely considered to be a 'blunt tool' for the control of appropriate behavioural and physiological responses as their presence increases the probability an appropriate response to a situation will occur but do not guarantee it (Goymann and Hofer 2010). The neuropeptide hormone oxytocin is crucial for initiating and modulating maternal and social behaviour in vertebrates (reviewed in Gimpl and Fahrenholz 2001). Oxytocin is released by a variety of stimuli, including scent, sight, touch and sound of a dependant infant or bond partner (reviewed in Uvnäs-Moberg, 1998). It promotes care-giving and pro-social behaviours (humans, Kosfeld *et al.* 2005 and reviewed in Ross and Young 2009) by providing a physiological reward for their expression by activating reward and pleasure pathways in the brain (Baskerville and Douglas 2010). Oxytocin has been shown to be essential for recognition abilities (Ferguson *et al.* 2000 and 2001) and the formation of the mother-offspring bond (Lévy *et al.* 1995, Da Costa *et al.* 1996, Dwyer 2008) in a variety of species. Critically in species that rely on olfactory recognition of offspring such as sheep, oxytocin is responsible for triggering a limited period of heightened sensitivity in the olfactory bulb for 1-4 hours after parturition to allow mother-offspring bonds to form (Alexander *et. al.* 1986, Lévy *et al.* 1995, Kendrick *et al.* 1992, Lim and Young 2006).

The physiological qualities of oxytocin make it a potentially important hormone for research into the regulation of maternal behaviour in grey seals. Olfactory recognition is widely theorised as a crucial modality for phocid mothers to recognise their pups (Insley *et al.* 2003) and has been shown to be important in one species of pinniped, the Australian sea lion (*Neophoca cinerea*) (Pitcher *et al.* 2011). Additionally peripheral oxytocin concentrations have been shown to be positively correlated with behaviours

such as lowered infanticide in mice (McCarthy 1990), increased aggression towards foreign offspring in sheep (Kendrick 2000) and an increased number of checks a mother makes on her infant in humans (Broad *et al.* 2006, Feldman *et al.* 2007), all behaviours that can occur in maternal grey seals on a breeding colony (see Chapter 4.). Therefore, the hormone appears to be an ideal candidate for the control of a wide range maternal behaviour displayed by grey seal mothers.

In humans patterns of basal oxytocin concentrations throughout the year appear to follow the Challenge Hypothesis (Wingfield *et al.* 1990), which describes levels of endocrine activity using testosterone as a case study. Level 'A' represents concentrations found in non-breeding animals, level 'B' is the elevated, breeding basal concentration of the hormone required to trigger essential reproductive behaviours, and level 'C' is denoted a 'maximum response' concentration. Level C contains the highest peak concentrations of the hormone that occurs from transitory stimuli such as male-male aggression. While oxytocin expression throughout different life history stages of a species has not been considered in the context of the Challenge Hypothesis, existing data on human plasma concentrations support this pattern of expression for this hormone and may provide a useful framework for identifying critical failures in the expression of maternal behaviour.

Oxytocin and its role in promoting maternal behaviour and bonding are widely conserved across the mammalian class (Gimpl and Fahrenholz 2001). We therefore predict that a similar pattern of plasma oxytocin concentrations will occur in female grey seals with dependant pups as in human, with a 'non breeding basal' (level A), a 'breeding basal' (level B) and a suckling peak (level C). However, if a positive mother-pup bond does not form between the two, we hypothesise that level B, the breeding basal level, will not be achieved, resulting in maladaptive behaviour expression in the mother during that season regardless of her prior success or experience in raising pups. Additionally, we will investigate whether plasma concentrations in breeding mothers correlate to any behavioural indices during their time with their pups. The natural frequencies of maternal behaviour as discussed in Chapter 4. will be analysed with plasma oxytocin concentrations sampled twice throughout the dependant period. This study represents the first work measuring and documenting the plasma concentrations of oxytocin in any marine mammal species, therefore there is no basal dataset or figures to provide a meaningful comparison to breeding concentrations. We therefore must

generate these data ourselves, investigating the concentrations found in all life stages of the grey seal and any additional phocid species that we can gain access to.

The objectives of this chapter were to:

1. Define basal concentrations of plasma oxytocin in phocid seals, covering the main life history stages of our study species, the grey seal.
2. Determine if the pattern of oxytocin release throughout a grey seal's life history fits the concept described in the Challenge Hypothesis and correlate any behavioural extremes to the pattern observed.
3. Correlate the variation in plasma oxytocin concentrations of grey seal mother's with the variation in maternal behaviour they exhibited while with a dependant pup.
4. Correlate the variation in dependant pup plasma oxytocin concentrations with the variation in behaviour they exhibited while with their mothers.

5.2. Methods

5.2.1. Defining basal concentrations of plasma oxytocin in phocid seals

Study animals

Three species of phocid seal were utilised for plasma collection in this study. Along with the focal species (grey seal), samples from adult non-breeding and juvenile harbour seals and adult non-breeding Weddell seals were available.

Grey seal plasma samples were obtained from adult breeding grey seal females with a dependant pup ('mothers') from the North Rona and Isle of May colonies between September and December, 2010 and 2011. The total number of mother-pup pairs sampled was 74, with some females recaptured in both years of the study (n=22). In addition to these, a 'non-breeding' sample set was collected from 14 (nine female, five male) non-breeding juveniles on the Isle of May colony in 2011. Finally a large dataset of weaned grey seal pup plasma samples (n=113) was collected from the North Rona colony in 2009 (n=8), 2010 (n=13) and 2011 (n=15) and from the Isle of May in 2010 (n=29) and 2011 (n=48).

Harbour seal samples were collected from adult, non-breeding, free ranging individuals caught as part of telemetry projects from 2010 - 2011 around the coast of Scotland (n=23). Seals were captured from small haul out groups containing 10-50 individuals. Additional samples were taken from one adult male and two juvenile (two male, one female) seals that were captured from the Eden River in Fife, Scotland and held during 2011 at the Sea Mammal Research Unit pool facility in St Andrews, Scotland (see Chapter 2. for more details).

Weddell seal samples were collected from adult, non-breeding, free-ranging individuals caught as part of a telemetry project in the Antarctic during 2011 (n=23). Animals were captured from ice floes during individual haul outs.

Plasma sampling and analysis

Plasma samples were collected and analysed using the veinipuncture and laboratory methodology outlined in Chapter 2. However the capture and restraint methodology varied with species, study site and age class. All adult female grey seals from a mother-pup pair, Weddell seals and the captive adult harbour seal were captured and restrained for sampling using the protocol outlined in Chapter 2. using chemical and manual restraints. All juvenile non breeding individuals, weaned pups and dependant pups were also sampled as described in Chapter 2, using only manual restraint.

Free ranging adult harbour seals were caught using a variety of techniques dependant on the particular haul out site the seals had chosen. Both 'rush and grab', which involved a rapid approach to a haul out using a Zodiac and manually catching individuals in hoop nets, and 'pop up nets', which involved remotely deployed, submerged nets around a haul out, were used to capture and restrain individuals. Study animals were then drugged with Zoletil® while in the net just before any procedures such as blood sampling and tag applications were performed.

All samples were then stored, processed and analysed as outlined in Chapter 2. Inter-assay coefficient of variance across the 16 ELISA plates used in this study was 6.1%.

Statistical analyses

All analyses were performed using the statistical package R 2.15.0 (R Development Core Team 2012). Differences according to species, sex and any interaction between the two was examined by a two-way ANOVA after the data were transformed using

reciprocal square roots to establish normality and eliminate non-homogenous variances in the data.

Mother-pup pair grey seal samples were first analysed to determine if a mean of the early/late samples could be used in subsequent analyses. The intraclass correlation coefficient (ICC) for both mother and pup oxytocin concentrations across early and late lactation were calculated and two Kruskal Wallis tests examining sample timing during lactation (early and late) and plasma oxytocin concentrations was performed. A GLM was then used to examine the variation in plasma oxytocin concentration with age class (dependant pup, weaned pup, non breeding and mothers) and biologically plausible explanatory variables including sex, colony (Isle of May or North Rona) and year of sampling (2009, 2010 and 2011) using the multiple generalized cross validation library mgcv (Wood 2012) after a natural log transformation of the oxytocin concentrations to ensure equal variances in the data. Gaussian error distribution was used and model selection was performed using backwards stepwise elimination through examination of AIC values, QQ and residual plots to identify the best model for the data (Richards 2005).

5.2.2. Determination of whether plasma oxytocin concentrations follow the pattern of expression described in the Challenge Hypothesis

Study site

All individuals used in this part of the study were from the North Rona or Isle of May colonies. North Rona samples were collected from the 3rd October – 31st October 2010 and 2nd October – 2nd November 2011, and on the Isle of May between 28th October – 12th December 2010 and 30th October – 3rd December 2011.

Study animals and categorisation

Non-breeding individuals (level A), mothers (level B) and mothers nursing their pups (level C) were used to represent the three levels of the challenge hypothesis. The level A sample set was collected from 14 (nine female, five male) juveniles on the Isle of May colony in 2011. These individuals were all between 12 and 24 months of age to ensure they were non-breeding animals, as the earliest record of grey seals reaching sexual maturity is 3 years (Hammill and Gosselin, 1995). 74 mothers with dependant pups were sampled to generate the level B dataset, with some females recaptured in both

years of the study (n=22). Two females were sampled while sucking their pups to generate data points for level C.

To investigate any relationship between the levels described above and success/failure at raising a pup in a breeding season, mothers were then split into two groups depending on their observed behaviour for that breeding season, classed 'good' and 'bad' mothers. These groups are defined as:

'Good' mothers: exhibited maternal care towards the pup for at least 16 days, the pup achieved a mass of over 30kg at time of weaning and the pup was alive at weaning (n=66).

'Bad' mothers: exhibited no maternal care towards the pup, or only exhibited care-giving behaviour for a short duration of time (<4 consecutive days since birth) (n=5), including individual's whose pup died inexplicably during the dependant period (n=2). Additionally, individuals that invested in non-filial offspring were included in this category (n=1). Total sample size, n=8.

Plasma sampling and analysis

Plasma samples were collected and analysed using the veinipuncture and laboratory methodology outlined in Chapter 2. The constraints of the capture techniques used when handling adult female seals made obtaining plasma samples from natural suckling bouts for the level C dataset difficult. Oxytocin has a rapid clearance rate in plasma (0.027 L/kg/min in human men and 0.021L/min/kg in human women, Leake *et al.* 1980), and nursing peaks in rats have been shown to rapidly return to basal concentrations (Higuchi *et al.* 1985). Therefore, if nursing peaks in seals are comparable to those found in nursing humans (53.2-224pg/ml, Dawood *et al.* 1981 and 13-54pg/ml, Drewett *et al.* 1982), the 10 minute wait for the anaesthetic drug to immobilise the mother would eliminate or greatly reduce any oxytocin peaks triggered by pre-capture nursing in the plasma. This problem could not be solved in any way through experimental design, as mothers cannot be sampled without the anaesthetic and pups will not feed in the presence of people. The two instances where samples were obtained from suckling mothers occurred as she became immobile with her ventral surface exposed and her pup chose to initiate a suckling bout during the ten minute wait for the female to become immobilised (Figure 5-1), offering two opportunities to obtain these important samples. In these two cases, the suckling bout was allowed to finish naturally and was followed

by the immediate capture and sampling of the female to minimise the oxytocin lost to natural clearance of the hormone from circulation.

All samples were then stored, processed and analysed as outlined in Chapter 2. Inter-assay coefficient of variance across the 16 ELISA plates used in this study was 6.1%.

Figure 5-1. A female grey seal nursing her pup during the 10 minute period for the anaesthetic drug to take effect, enabling the collection of a plasma sample during the suckling event. The dart that administered the drug intramuscularly is clearly visible in her left flank.

Statistical analysis

All analyses were performed using the statistical package R 2.15.0 (R Development Core Team, 2012). An independent 2-group t-test with equal variances was used to look for any differences in plasma oxytocin between the level A and level B categories. The two data points for level C were not included in the analysis due to the low sample size. A one way ANOVA was used to test for differences between the two maternal behaviour categories (good and bad mothers) and the non-breeding group (basal). The data were analysed after a natural logarithmic transformation as the original data were not normally distributed when submitted to a Shapiro Wilkes test ($p<0.001$).

5.2.3. Correlating variation in plasma oxytocin concentrations and maternal behaviour in grey seal mothers

Study sites

Detailed behavioural observations were carried out alongside plasma sampling on the North Rona breeding colony between 3rd October – 31st October 2010 and 2nd October – 2nd November 2011.

Study animals

Mothers that were clearly visible on the North Rona colony had detailed behavioural observations taken in tandem with plasma sampling. The main colony site on North Rona has several gullies crossing it where mothers can pup out of sight from the hides. In 2010 there were ten such mothers and in 2011 there were eight, four of whom were sampled and observed in both study years.

Behavioural observations

The ethogram described in Chapter 2. was used to record behavioural data using a scan sampling protocol while in the field, and the PCA components identified in Chapter 4. were used to collate the various categories of behaviours observed into five that account for the largest amount of variance in the data.

Plasma sampling and analysis

Two plasma samples were taken from all mothers, during early and late lactation in the 18 day dependant period. The first was 1-7 days after the pup's birth and the second was approximately 10 days after the first sampling event. Samples were collected using the capture, restraint and blood sampling methodology outlined in Chapter 2.

Statistical analysis

Basal oxytocin concentrations were calculated for free ranging mothers during early and late lactation from the North Rona colony in 2010 (n=10) and 2011 (n=8). Detailed behavioural observation data recording the percentage of observed time spent in each behaviour from these mothers in early and late lactation was collated into the four components described in Chapter 4 using PCA, with an additional behavioural component for 'resting'. The mother-pup distance (the mean distance a mother

maintained from her pup in adult body lengths) was also a variable in the analysis. This gave a total of six behavioural variables to incorporate into subsequent models for this chapter (Table 5-1).

Table 5-1. The behavioural components accounting for 80% of the variation in the behavioural data exhibited by mothers on North Rona in 2010 and 2011 and the behavioural groupings (from the methods section of Chapter 4) they include.

Component name	**Behavioural groupings included**
Awareness of pup	Checking pup and locomotion
Pup care	Nursing and interacting with pup
Aggression	Aggression
Alertness	Alert to surroundings, not including pup
Maternal resting	Resting

GAMs (Wood 2006a) were used to analyse the explanatory variables affecting mother-pup distance and percentage time spent performing each behaviour component, resulting in six models. Biologically plausible explanatory variables included in these models were plasma oxytocin concentration for mothers, plasma oxytocin concentration for pups, date of the pup's birth, sample timing during the season (early or late lactation), the pup's sex, the rate a mother lost mass across the dependant period (kg/day), the rate the pup gained mass during the dependant period (kg/day) and the mother/pup distance in the five models where this was not the dependant variable being tested (see Appendix A, Table A-1). Plasma oxytocin for both the mother and pup were included as this was the main question this chapter addressed. The morphometric variables were included as date of pup birth, pup sex, time of behavioural/plasma sampling in lactation and rates of maternal mass loss/pup mass gain have been shown to influence each other and the behaviour exhibited by individuals on a breeding colony (Fedak and Anderson 1982, Pomeroy *et al.* 1999). Models were fitted using the multiple generalized cross validation library mgcv (Wood, 2012). The identities of mothers were fitted as a random effects smooth (Wood 2006b) to control for pseudo-replication in the dataset from using some of the same individuals over the two years of the study. The

smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). Three of the six models were fitted with a Gamma error distribution with log links, one was fitted with a poisson error distribution with log links and two were fitted with Gaussian error distributions. Model selection was done by backwards stepwise elimination through examination of R^2 values, AIC values, QQ and residual plots to identify the best model for the data. In the model of the mother-pup distance, the selection process lead to the elimination of the smooth for mother's identity, and the model became a generalised linear model (GLM).
One additional GAM with the mother's plasma oxytocin concentration as the dependant variable was run. This analysis was performed as at this stage in the thesis the data collected allows only correlatory analyses, which cannot indicate causality, and some behaviours such as nursing cause oxytocin release in other mammalian species (reviewed in Uvnäs-Moberg *et al.* 1998). Explanatory variables included in this model were plasma oxytocin concentration for pups, date of the pup's birth, sample timing during the season (early or late lactation), the pup's sex, the proportion of time spent suckling or interacting with the pup, the rate a mother lost mass while suckling her pup (kg/day), the rate the pup gained mass while suckling (kg/day) and the mother/pup distance. Mother identity was fitted as a random effect smooth using maximum likelihood and the model was fitted with a Gamma error distribution with log links. Model selection was performed as described above (see Appendix 1, Table A-3).

5.2.4. Correlating variation in plasma oxytocin concentrations and pup behaviour in dependant grey seal pups

Exactly the same methodology was used to obtain and analyse plasma samples and behavioural data from the 18 dependant pups of the mothers described above in the methods in section 5.2.3 of this chapter.

Statistical analysis
Basal oxytocin concentrations were calculated for free roaming dependant pups during early and late lactation from the North Rona colony in 2010 (n=10) and 2011 (n=8). Detailed behavioural observation data from these pups in early and late lactation was combined into the four components described in Chapter 4. using PCA, with an additional category for 'resting' due to the strong negative loading of this aspect of

behaviour in the PCA in Chapter 4. compared to the other four behavioural categories (Table 5-2).

Table 5-2. The behavioural components accounting for 80% of the variation in the behavioural data exhibited by dependant pups on North Rona in 2010 and 2011 and the behavioural groupings (from the methods section in Chapter 4) they include.

Component name	**Behavioural groupings included**
Interaction with mother	Suckling and interacting with mother
Pup activity	Locomotion and alertness to surroundings, not including mother
Play behaviour	Playing and exploring
Awareness of mother	Checking mother
Pup resting	Resting

GAMs (Wood 2006a) were used to analyse the percentage time spent performing each behaviour across lactation for all five behavioural components, resulting in five models. Biologically plausible explanatory variables included in these models were plasma oxytocin concentration for mothers, plasma oxytocin concentration for pups, date of the pup's birth, sample timing during the season (early or late lactation), the pup's sex, the rate a mother lost mass during the dependant period (kg/day), the rate the pup gained mass during the dependant period (kg/day) and the mother-pup distance in adult body lengths in the five models where this was not the dependant variable being tested (see Appendix A, Table A-2). Models were fitted using the multiple generalized cross validation library mgcv (Wood, 2012). The identities of mothers were fitted as a random effects smooth (Wood 2006b) to control for pseudo-replication in the dataset from using some of the same individuals over the two years of the study. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). One of the six models was fitted with a Gamma error distribution with log links based on the model selection criteria (see below), two were fitted with a poisson error distribution with log links due to zero inflation of the data and two were fitted with Gaussian error distributions. Model selection was done by

backwards stepwise elimination through examination of R^2 values, AIC values, QQ and residual plots to identify the best model for the data (Richards 2005).
One additional GAM with the pup's plasma oxytocin concentration as the dependant variable was run. Explanatory variables included in this model were plasma oxytocin concentration for mothers, date of the pup's birth, sample timing during the season (early or late lactation), the pup's sex, the proportion of time spent suckling or interacting with the pup, the rate a mother lost mass during the dependant period (kg/day), the rate the pup gained mass during the dependant period (kg/day) and the mother/pup distance. Mother identity was fitted as a random effect smooth using maximum likelihood and the model was fitted with a Gamma error distribution with log links. Model selection was performed as described above (see Appendix A, Table A-3).

5.3. Results

5.3.1. Defining basal concentrations of plasma oxytocin in phocid seals

Basal concentrations of plasma oxytocin in non-breeding grey, harbour and weddell seals

A two way ANOVA showed there was significant differences in plasma oxytocin concentrations across the three phocid species analysed (grey, harbour and Weddell) ($F_{2, 57} = 11.1$, $p<0.001$) but there was no difference between the sexes ($F_{1, 57} = 0.4$, $p=0.5$) and no interaction between the species and sex of the individual ($F_{2, 57} = 1.7$, $p=0.2$). A Tukey honest significant differences post-hoc test was performed to determine which species were different. Plasma oxytocin concentrations in grey seals (Hg, mean= 4.2 ±0.4 pg/ml) were not significantly different to harbour (Pv, mean= 6.1 ±0.55 pg/ml, $p=0.07$) or Weddell (Lw, mean= 3.8 ±0.3 pg/ml, $p=0.2$) seals. Plasma oxytocin concentrations were higher in harbour seals compared to Weddell seals however ($p<0.001$) (Figure 5-2).

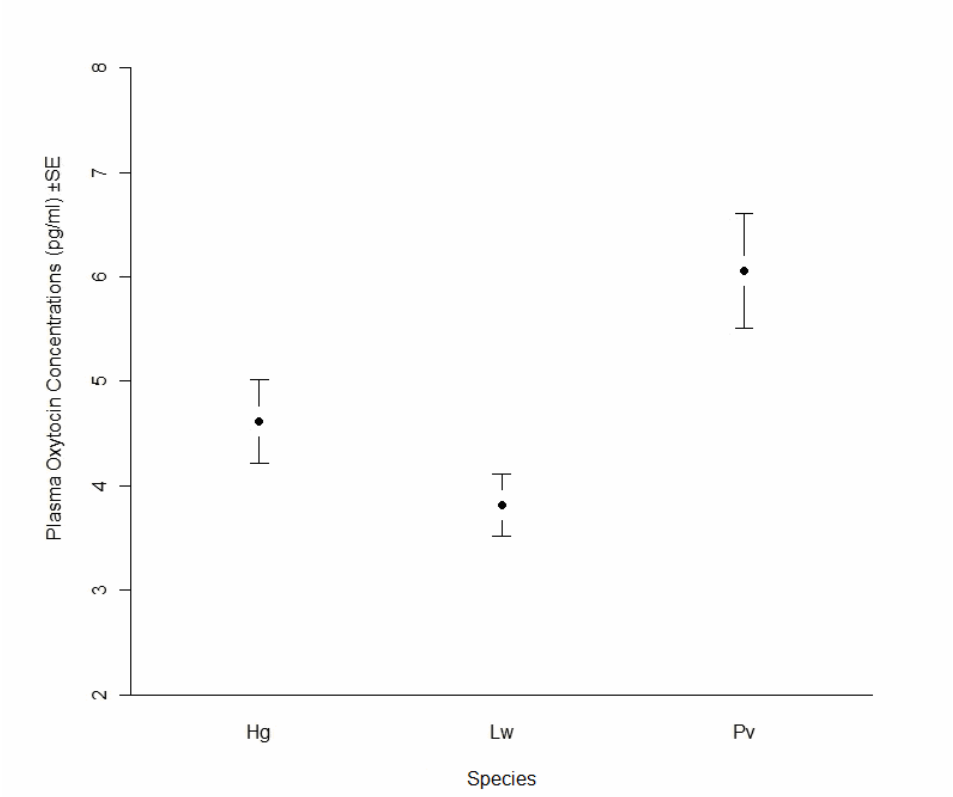


Figure 5-2. Mean plasma oxytocin (pg/ml) in the three phocid seal species analysed, grey (Hg), Weddell (Lw) and harbour (Pv) seals with standard error bars.

Plasma concentrations in grey seals of different life history states

There were no significant differences between early and late lactation oxytocin concentrations for either mothers ($F_1 = 0.61$, $p = 0.6$) or pups ($F_1 = 1.2$, $p = 0.3$). ICC analyses additionally showed significant positive agreement across individuals that were sampled in early and late lactation for both mothers ($ICC_{63} = 0.23$, $p = 0.03$) and pups ($ICC_{58} = 0.55$, $p < 0.001$) (Figures 5-3 and 5-4). Therefore in all subsequent analyses for this section of the chapter (Defining basal concentrations of plasma oxytocin in phocid seals) a mean of the early and late oxytocin concentration was used for each individual in the study for mothers and pups.

In grey seals the GLM showed that of the four potential explanatory variables, only the life history stage significantly affected plasma oxytocin concentration (Figure 5-5). Sex ($p=0.4$), colony ($p=0.2$) and year of sampling ($p=0.8$) did not impact on plasma oxytocin in any life history stage (Table 5-3). Pups had the highest plasma oxytocin concentrations (21.9 ±2.2pg/ml) while mothers (7.5 ±0.75pg/ml) and weaned pups (10.2 ±1pg/ml) had higher levels than non-breeding individuals (4.8 ±0.75pg/ml).

Table 5-3. Fixed effect variables from the GLM of plasma oxytocin concentrations of non-breeding, juvenile grey seals compared to grey seal mothers, dependant and weaned pups, their estimates, standard errors and p values

Explanatory Variable	**Estimate**	**Standard Error**	**P value**
Class (Mother)	0.4	0.1	<0.001
Class (Pup)	1.5	0.1	<0.001
Class (Weaned pup)	0.7	0.1	<0.001
Sex	0.04	0.05	0.4
Colony	0.06	0.05	0.2
Year	0.01	0.04	0.8

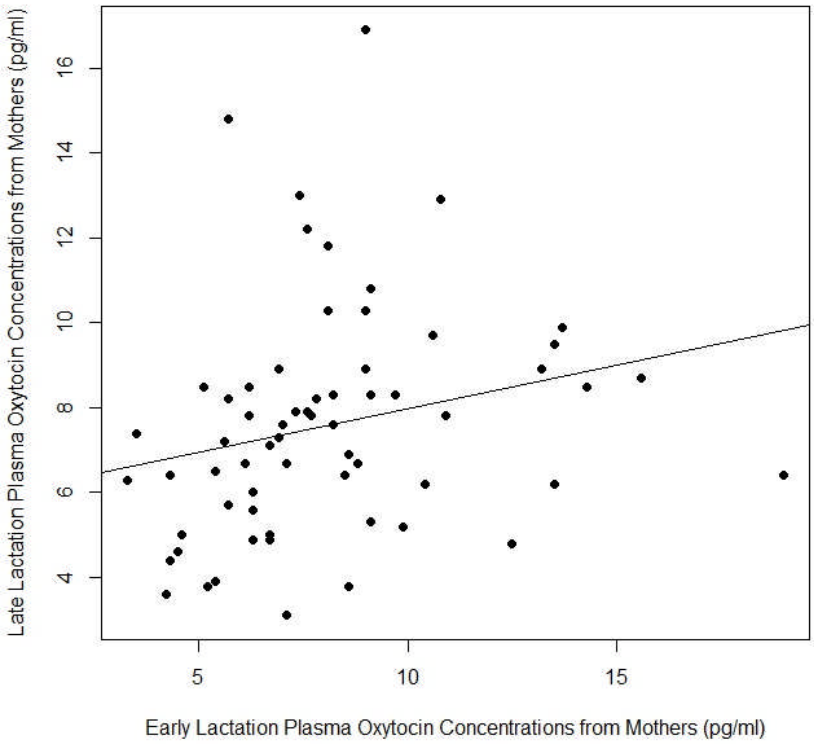


Figure 5-3. Plasma oxytocin concentrations (pg/ml) taken from individual mothers in early and late lactation with the line of best fit.

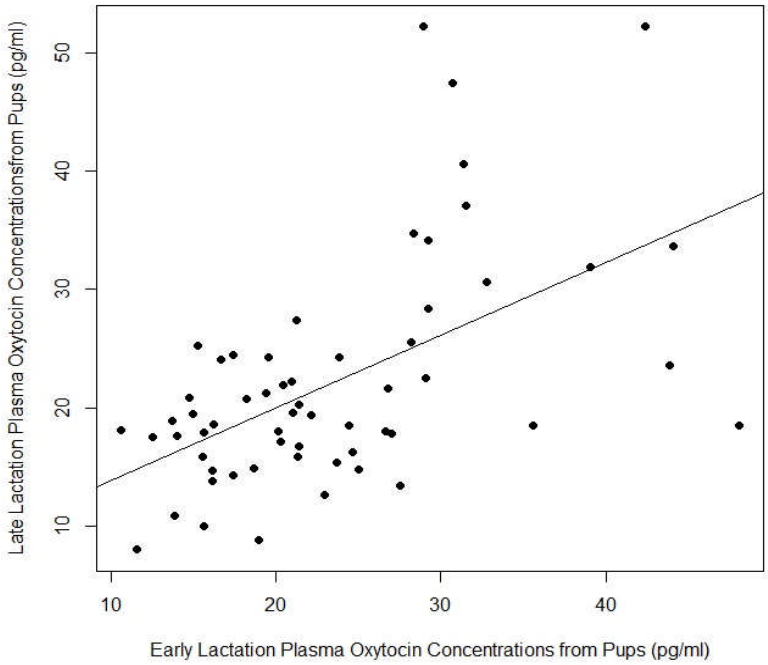


Figure 5-4. Plasma oxytocin concentrations (pg/ml) taken from individual dependant pups in early and late lactation with the line of best fit.

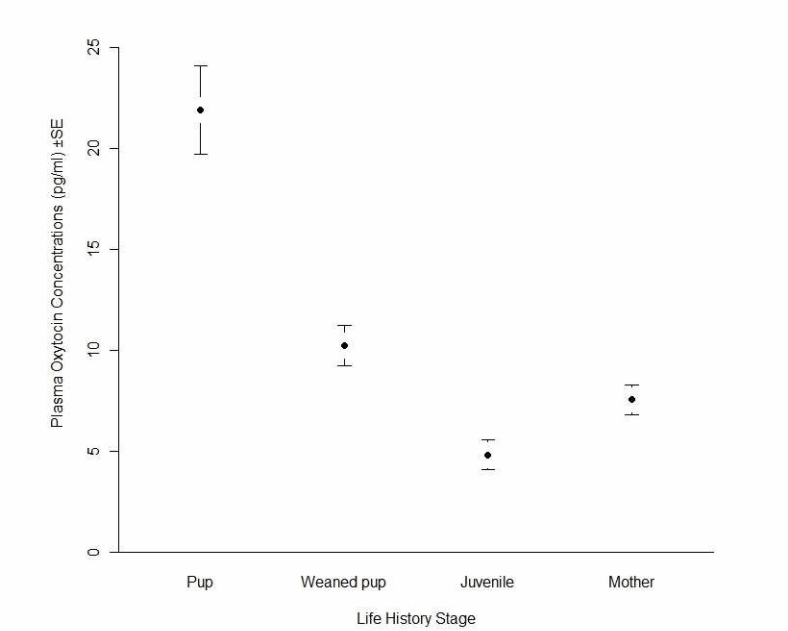


Figure 5-5. Mean plasma oxytocin concentrations (pg/ml) across four life history stages in grey seals, dependant pups (Pup), weaned pups (Weaned pup), non-breeding juveniles (Juveniles) and mothers with dependant pups (Mother) with standard error bars.

5.3.2. Determination of whether plasma oxytocin concentrations follow the pattern of expression described in the Challenge Hypothesis

An independent two-group t-test with equal variances showed non-breeding individuals (level A) had lower plasma oxytocin concentrations (4.8 ±0.5pg/ml) than mothers with dependant pups (level B) (7.5 ±0.75pg/ml)
(t_{86}= -4.1, $p < 0.001$). As there were only two data points representing nursing mothers (level C), these were not included in the statistical analysis. These concentrations were 30.5pg/ml and 39.8pg/ml, five times higher than level B (Figure 5-6).
A one way ANOVA was used to test for differences across the two ('bad' and 'good') maternal behaviour categories and the non-breeding basal group
($F_{(2, 85)} = 14.21$, $p<0.001$) and a Tukey honest significant differences post-hoc test was performed to determine which treatments were different. Plasma oxytocin concentrations were significantly higher in the 'good' category (7.8 ±1.2 pg/ml) when compared to basal (4.8 ±1.1 pg/ml, $p<0.001$) and 'bad' individuals (5.1 ±0.85 pg/ml,

p =0.006) concentrations. There was no significant difference between concentrations in individuals in the 'bad' category and basal group (p=0.9) (Figure 5-7).

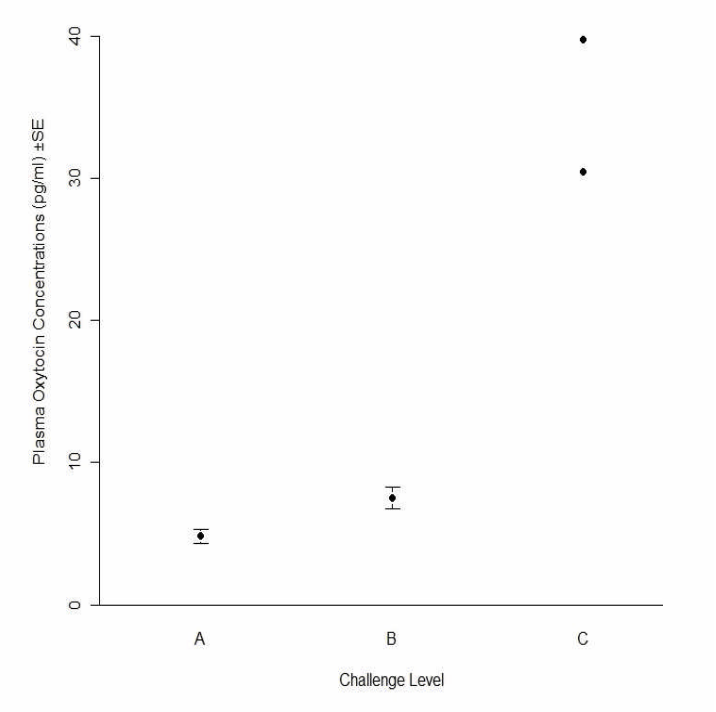


Figure 5-6. Error bar plot (for A and B) showing the mean plasma oxytocin concentrations (pg/ml) across the three proposed levels of the challenge hypothesis, including the non-breeding basal (level A), the breeding basal from mothers with pups (level B) and the maximum response from two mothers sampled while nursing their pups (level C).

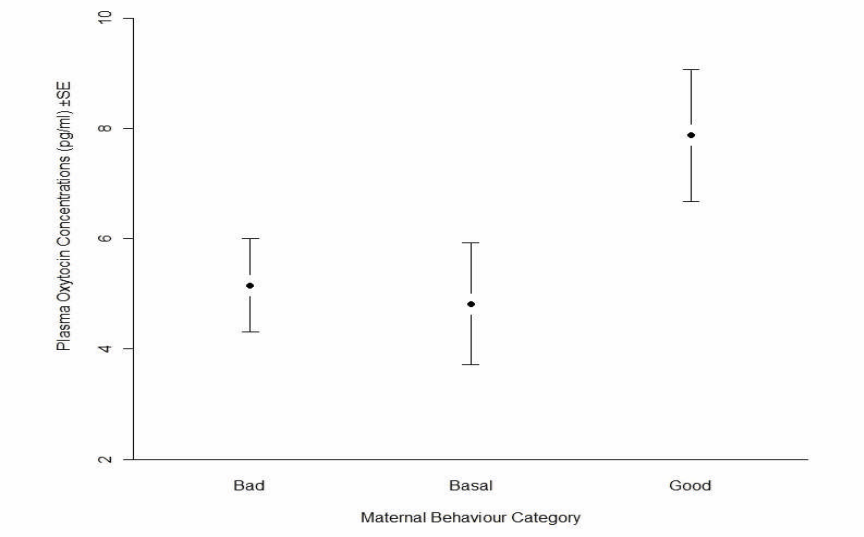


Figure 5-7. Mean plasma oxytocin concentrations (pg/ml) in mothers classed as either 'good' or 'bad' for a breeding season and the basal concentration from non-breeding individuals with standard errors.

5.3.3. Correlating variation in plasma oxytocin concentrations and maternal behaviour in grey seal mothers

Across the five GAMs and single GLM for the frequencies of behaviour exhibited by mothers with dependant pups throughout lactation (n=36), plasma oxytocin concentration was a significant explanatory variable in two of the models (see Appendices A, Table A-4 and A-5).

The percentage time spent performing pup checks and the amount of activity a mother exhibits ('Awareness of pup behaviour' from Chapter 4.) was positively correlated with the plasma oxytocin concentrations in that female ($p=0.01$), while additionally being affected by the sex of the pup ($p=0.01$). The mean distance between a mother and pup was negatively correlated with plasma oxytocin concentrations ($p=0.05$) and was negatively correlated with the time during lactation ($p=0.02$) (Table 5-4).

For both 'resting' and 'Pup care' behaviours (combining percentage time spent nursing and interacting with the pup), the only significant explanatory variable was the time during lactation, with mothers resting less ($p=0.001$) and spending more time nursing and interacting with their pup ($p=0.009$) in late lactation than early lactation. The components combining aggression behaviour and alertness behaviour were not correlated with any of the variables included in the models.

In the GAM analysing maternal plasma oxytocin, maternal concentrations were positively correlated with the pup's plasma oxytocin ($p=0.002$) (Table 5-4 and Appendix A, Tables A-4 and A-5).

Table 5-4. Significant fixed effect variables from the GAMs and GLM for mother behaviour where plasma oxytocin concentrations significantly impacted on the dependant variable, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Awareness of pup behaviour	Maternal plasma oxytocin concentration (pg/ml)	0.04	0.01	0.01
	Pup sex (male/female)	-0.33	0.12	0.01
Mean mother/pup distance	Maternal plasma oxytocin concentration (pg/ml)	-0.09	0.05	0.05
	Time during lactation (early/late)	-0.77	0.31	0.02
Maternal plasma oxytocin concentration	Pup plasma oxytocin concentration (pg/ml)	0.02	0.006	0.002

5.3.4. Correlating variation in plasma oxytocin concentrations and pup behaviour in dependant grey seal pups

Across the five GAMs looking at the frequencies of behaviour exhibited by pups throughout lactation (n=36), plasma oxytocin concentration was a significant explanatory variable in one of the models (see Appendix A, Table A-6 and A-7). The percentage of time spent in 'playing' behaviour was significantly positively correlated with the plasma oxytocin concentrations in that pup (p=0.02) and to the pup's date of birth in the season (p=0.02) while the time during lactation was significantly negatively correlated (p=0.02) (Table 5-5).

For both 'resting' and 'interaction with mother' behaviours (combining percentage time spent suckling and interacting with the mother), plasma oxytocin concentrations were not a significant explanatory variable. The only significant explanatory variable in both models was the rate of maternal mass loss, which was negatively correlated with resting (p=0.04) and positively correlated with 'interaction with mother' behaviours (p<0.001). The component combining 'pup activity' behaviours (percentage time spent alert to the surroundings and active) was affected by the time during lactation (p=0.05), the mother-pup distance (p=0.05) and the maternal plasma oxytocin concentration (p=0.003) with pups in late lactation being less active, and additionally were less active when

mother/pup distances were small and with a mother with high plasma oxytocin (Table 5-5). The component combining 'awareness of mother' behaviour (checking the mother) was not significantly affected by any of the variables we included in the models.

In the GAM analysing pup plasma oxytocin, pup concentrations were positively correlated with maternal concentrations ($p<0.001$) and were negatively correlated with the rate of mass loss their mother experienced in kg/day ($p=0.005$), while also being affected by the individual identity of the mother ($p<0.001$) (Table 5-5 and Appendix, Table A-6 and A-7).

Table 5-5. Significant fixed effect variables from the four GAMMs concerning pup behaviour were plasma oxytocin concentrations significantly impacted on the dependant variable, their estimates, standard errors and p values.

Dependant variable	**Explanatory variable**	**Estimate**	**Standard Error**	**P value**
Play behaviour	Pup plasma oxytocin concentration (pg/ml)	0.18	0.08	0.02
	Time during lactation (early/late)	-3.98	1.37	0.007
	Pup birth date (day in October)	0.45	0.18	0.02
Pup plasma oxytocin concentration	maternal plasma oxytocin concentration (pg/ml)	0.06	0.01	<0.001
	rate of maternal mass loss (kg/day)	-0.19	0.07	0.005
	mother identity (smooth term)	NA	0.16	<0.001

5.4. Discussion

5.4.1. Defining basal concentrations of plasma oxytocin in phocid seals

Basal concentrations of plasma oxytocin in non-breeding grey, harbour and Weddell seals

Using the protocol developed in Chapter 3, plasma samples from three species of phocid seals were successfully analysed, providing crucial basal datasets from non-breeding individuals to compare against any samples gathered from seals of different life history stages. Non-breeding, juvenile grey seals had comparable plasma concentrations to those found in non-breeding, adult Weddell and harbour seals; however concentrations in Weddell seals were significantly lower than those found in harbour seals. While it is not impossible that the two species genuinely have different basal oxytocin concentrations to each other, the fact that the grey seal samples are not different to either datasets and appear to span the middle ground between the two indicates it is more likely that one or both of the Weddell or harbour seal sample sets are biased towards the lower or higher range respectively of the basal range. One study prior to this has tested for differences between adults of different species; however the study design was focused on species comparisons between strongly contrasting social behaviour (e.g. highly social versus solitary vole species, Kramer *et al.* 2004). Other studies examining adult individuals typically focus on one species between different life history periods (e.g. pregnant/early labour/late labour in humans, Dawood *et al.* 1981). Additionally the varied analytical methods used to detect and measure plasma oxytocin in the literature make cross study comparisons difficult (see Chapter 3.).

The mechanism for such a bias to enter the datasets is most likely to be the different capture methodologies used to obtain the samples from Weddell and harbour seals. Oxytocin is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis during extreme stressors (Cook 1997, Devarajan and Rusak 2004, Tops *et al.* 2007), and plasma oxytocin has been documented to rise when given cortisol manipulations (humans**,** Tops *et al*. 2012) or in response to some experimental procedure stressors (such as physical restraint over several hours in rodents, Grippo *et al.* 2009, Pournajafi and Carter, unpublished observations). The capture protocol used to obtain the harbour seal plasma samples would typically involve a mass capture of several study animals at once, necessitating that some were restrained and had to wait while each individual

underwent the sampling and tagging procedure in turn. Of the 23 individuals that made up the harbour seal database, 10 were physically restrained in hoop nets after capture for over one hour, which may have generated some plasma oxytocin concentrations that were in a higher range than those sampled closer to the initial capture. Unfortunately, the data collected on the time intervals between captures and plasma sampling of these individuals was not of sufficient resolution to directly test for this effect. In contrast to this, the Weddell seals were captured on an individual basis using chemical immobilisation and no physical restraints other than a bag over their heads. Researchers were able to capture and sample adult Weddell seals without physical restraints due to the subdued response to land predators and stressors this Antarctic species has (Boehme, pers. comm). These capture methodologies, while not producing samples significantly different from grey seals, are unlikely to generate many samples in the upper quartile of the detected basal oxytocin concentrations for phocid seals, and only one individual of the 23 sampled exceeded 5pg/ml. Therefore, the difference between harbour and Weddell seals is most likely due to the different capture methodologies and their responses to land based stressors rather than true functional differences between these two species.

Across all three species analysed, there were no sex differences in the basal plasma oxytocin concentrations. While to date there is only one study directly testing for sex differences in plasma oxytocin concentrations, there are many studies using male and female human plasma as control groups in behavioural and social bonding experiments. Such studies also report no significant differences between the sexes (Amico *et al.* 1981, Grewen *et al.* 2005, Marazziti *et al* 2006) which agrees with our findings. The results of the only study that directly tests for sex differences in plasma oxytocin concentrations of voles and rats found that females had consistently higher plasma oxytocin concentrations than males (Kramer *et al.* 2004). However the methodologies of this study make these results questionable due to the use of unextracted plasma in the analysis (see Chapter 3.). Therefore, our results agree with other studies using extracted plasma samples from males and females to form basal datasets, and, as there is no difference between non-breeding male and female individuals, both were included in the dataset in analyses for the rest of this thesis.

Plasma concentrations in grey seals of different life history stages

Non-breeding individuals have the lowest plasma oxytocin concentrations of the four life history stages analysed, while dependant pups have the highest. Mothers with dependant pups had concentrations significantly higher than non breeding individuals, but lower than newly weaned pups and dependant pups. There were no differences in oxytocin concentrations with sex, colony or sampling year, agreeing with the findings from the phocid species dataset from grey, harbour and Weddell seals (described above) and permitting the inclusion of both males and females from all study years in the analysis.

Plasma oxytocin concentrations in mothers of any species during the postpartum period are not well documented, however data on humans supports our finding that mothers with dependant young have elevated plasma oxytocin concentrations compared to non-breeding individuals. Reported concentrations for human mothers with dependant babies (10.8pg/ml, Dawood *et al.* 1982 and 5.4pg/ml, Drewett *et al.* 1982) are higher than concentrations reported in non-breeding human adults (< 2pg/ml, Grewen *et al.* 2005, Szeto *et al* . 2011, Gossen *et al.* 2012), but no study to date has compared the two directly. Oxytocin is released both centrally and into circulation in response to a number of stimuli from a dependant infant in humans and rodents, including suckling, touch, sounds and sight of the infant and interacting with the infant (Lucas *et al.* 1980, McNeilly *et al.* 1983, Johnston and Amico 1986, Kendrick *et al.* 1988, Uvnäs-Moberg 1998, Matthiesen *et al.* 2001, Feldman *et al.* 2007 and Strathearn *et al.* 2009). Grey seal mothers are in close contact with their dependant pups through out the majority of the lactation period, typically keeping pups within 1.5 body lengths of them and excluding other conspecifics from this range (Twiss *et al.* 2003). Mothers on the North Rona colony in particular spend the vast majority of the dependant period attending their pups and do not typically return to sea during this time (Anderson and Harwood 1985). Mothers are hypothesised to recognise their pups by scent (Fogden, 1971 and Insley *et al.* 2003), have variable ability to recognise their pup's call (McCulloch *et al.* 1999 and McCulloch and Boness 2000) and nurse their pups multiple times a day approximately every three hours (Haller *et al.* 1996). All these stimuli have been reported to trigger central and peripheral oxytocin release (McNeilly *et al.* 1983 and Uvnäs-Moberg *et al.* 1993), and these stimuli combined with the near constant proximity of the dependant pup may be the cause of the elevated plasma concentrations throughout the dependant period compared to non-breeding individuals (Dwyer 2008). Such oxytocin release may

be functionally important to motivate mothers to attend to their pups, as mothers must ignore the urge to return to sea to feed during the lactation period and must minimise the time they spend away from their pup to bath in pools, which has thermoregulatory importance (Redman *et al.* 2001). Oxytocin, therefore, provides a physiological mechanism in mothers to link the presence of her own pup and interacting with it to the activation of reward and reinforcement regions of the brain (Lim and Young 2006) while additionally continuing to stimulate the expression of maternal behaviour in that individual (Kendrick 2000). How long the elevation of plasma oxytocin lasts is uncertain, in this study it was not possible to obtain samples from mothers within a day of, or during, the sudden weaning process as there are no outward signs that it is about to happen. If elevated oxytocin is needed to motivate a mother to stay with and nurture her pup, then this must be eliminated if the weaning process is to take place at the end of the 18 day lactation period.

Only one previous study has noted that dependant infants have elevated plasma oxytocin concentrations compared to adults of the same species. Leake *et al.* (1981) reported that throughout a four day study period postpartum, dependant human babies had higher plasma oxytocin concentrations than adults, but was unable to provide any explanation for this result. Interestingly human children that are weaned from their mothers but still dependant on their parents for care appear to have similar plasma oxytocin concentrations to adults (children aged between 6-11 years: 1.2pg/ml (Modahl *et al.* 1998), adults: <2pg/ml (Grewen *et al.* 2005, Szeto *et al.* 2011 and Gossen *et al.* 2012)). This is in contrast to weaned grey seal pups, where plasma oxytocin concentrations are double that of non-breeding individuals despite having no contact with their mothers after the abrupt weaning process in this species (Boness and Bowen 1996). The high plasma oxytocin concentrations detected in dependant pups can be present for one of two reasons. The pups may be releasing the oxytocin due to appropriate stimuli from their mothers or they may be ingesting and absorbing oxytocin via the milk they drink during the dependant period. Ingestion of oxytocin from breast milk has been proposed in humans as a route of neonatal exposure to this hormone (Uvnäs-Moberg 1998, Carter 2003) but physical barriers and potential chemical degradation in the digestive tract (Fjellestad-Paulsen *et al.*1995, Prakash *et al.* 2009) make this route a questionable source of oxytocin. There are some buccal doses of oxytocin that have been shown to significantly raise plasma oxytocin concentrations in humans, however, the most successful manipulations are those with high doses applied

over more than one hour (Dawood *et al*. 1980, Ylikorkala *et al.* 1984, Landgraf 1985). As successful and failed doses of buccal oxytocin are known from these studies (Table 5-6), and the mean volume of milk a seal pup ingests in one day (3030ml/day, Iverson *et al.* 1993) has been determined for grey seals, we can use the mean oxytocin concentration in grey seal milk detected in Chapter 3. (147.1pg/ml) to calculate if seal pups do ingest quantities that have been shown to increase plasma oxytocin in one day or one suckling bout.

Table 5-6. Experimentally tested buccal doses of oxytocin for adult humans and their success rates.

Buccal dose given (units as stated in source)	Frequency administered	Total dose given in picograms	Successful?	Reference
70µg	Once	70,000,000pg	No	Landgraf 1985
200 IU	Every 20 minutes for 2 hours	2,400,000,000pg	No	Dawood *et al.* 1980
400 IU	Every 20 minutes for 2 hours	4,800,000,000pg	Yes (majority elevated to 24 - 50pg/ml)	Dawood *et al*. 1980

Using the concentration of oxytocin in phocid milk and the volume of milk a pup consumes in a day, grey seal pups ingest 445,713pg of oxytocin per day or 0.6% of the lowest dose that has been proven to have no effect on plasma oxytocin. Furthermore, this intake is split into approximately five suckling bouts in a 24 hour period (Pomeroy, pers. comm), meaning that on average in one suckling bout pups only consume 89,142.6pg of oxytocin, 0.1% of the failed dose. Pups would have to drink over fifty times the amount of milk they actually consume in a two hour period to approach the doses that have been proven to raise plasma oxytocin concentrations in humans. As pups ingest such a small amount of oxytocin during each suckling bout, it is unlikely

that this is the source of the high plasma oxytocin concentrations that are present throughout early and late lactation.

The other route for raising plasma oxytocin concentrations is endogenous release from the pituitary of the individual into circulation. As described above, oxytocin release is documented to occur both centrally and peripherally during interactions with, and close proximity to, an individual which one shares a positive social bond with, especially in mothers interacting with their offspring. If a pup is capable of recognising its mother and bonding with her, then the scent, sight, sound and tactile stimulation from her releases oxytocin peripherally (Nagasawa *et al.* 2012), and the close proximity the pair experience throughout the dependant period may trigger consistently high plasma oxytocin concentrations. The act of suckling and feeding has also been documented to raise plasma oxytocin concentrations in dependant and adult sows and dogs (Unväs-Moberg *et al.* 1985). In rats, oxytocin receptors in central brain regions develop on the first day postpartum (Shapiro and Insel 1989) and oxytocin itself is transcribed on the second day (Lipari *et al.* 2009), therefore, young dependant mammalian infants are able to produce oxytocin and respond to its presence (Wang and Young 1997). One study has documented rises in peripheral oxytocin in 4-6 month old human children interacting with their parents (Feldman *et al.* 2010b) and the large drop in pup peripheral oxytocin once the mother leaves the pup at weaning supports the idea that the mother's presence is maintaining the high plasma oxytocin concentrations. Elevated oxytocin concentrations would additionally motivate the pup to remain close to its mother, decreasing the likelihood of separation and disintegration of the mother-pup bond, which is cited as the biggest ultimate cause of pup mortality on a colony (Baker and Baker 1988). Behavioural evidence from two phocid seal species, the Weddell (Collins *et al.* 2005) and harbour seal (Renouf and Diemand 1984) also support the theory that pups play an active role in seeking out their mothers, maintaining close proximity to them and initiating suckling bouts, all behaviours that oxytocin promotes in other species (rats: Nelson and Panksepp 1996). Elevated oxytocin concentrations are important not only for promoting a pup to associate with its mother, but also because of the developmental effects oxytocin has on infants. Manipulations involving exposure to intraperitoneal oxytocin in neonatal female voles significantly affected the maternal and pair-bonding behaviour displayed subsequently as an adult (voles, Bales *et al.* 2007) and their reproductive success (voles, Cushing *et al.* 2005). Similar manipulations in neonatal male voles affected several reproductive fitness measures, such as sperm

transfer, sperm concentrations and sexual behaviour (Bales *et al.* 2004). Exposure to oxytocin during the neonatal period in rats can also cause higher body weight (Sohlström *et al.* 2000) and can additionally alleviate the negative effects in offspring of maternal malnutrition (Olausson *et al.* 2003). Therefore, the heightened plasma oxytocin concentrations experienced as a pup may have developmental consequences that affect the individual for its entire life, as well as promoting behaviours that increase the likelihood of the pup remaining with its mother.

5.4.2. Determination of whether plasma oxytocin concentrations follow the pattern of expression described in the Challenge Hypothesis

In grey seals basal plasma oxytocin concentrations fluctuate according to the pattern described in the Challenge Hypothesis. Non-breeding individuals have the lowest plasma oxytocin concentrations (level A), which become elevated to a breeding basal concentration when mothers are with their dependant pups (level B), and can additionally be influenced by behavioural stimuli to generate 'maximum response' peaks when nursing their pups (level C). The plasma concentrations of oxytocin detected in seals are comparable to those found in the different life history stages in humans. Non-breeding human adults (1.8pg/ml, Szeto *et al* .2011 and 1.7pg/ml, Gossen *et al.* 2012) and children (1.2pg/ml, Modahl *et al.* 1998) are lower than those of mothers with dependant babies (10.8pg/ml, Dawood *et al.* 1981 and 5.4pg/ml, Drewett *et al.* 1982) and peaks occur when mothers nurse their babies (53.2-224pg/ml, Dawood *et al.* 1981 and 13-54pg/ml, Drewett *et al.* 1982).

By dividing the individual mothers that made up level B into two groups defined by the overall outcome of that breeding season ('good' if the pup was raised to weaning and achieved >30kg mass, 'bad' if the pup died, was abandoned or the mother invested in non-filial offspring), it was possible to test whether there were any differences in oxytocin concentrations that may be related to the maternal behaviour expressed in a single breeding season. Bad mothers had plasma oxytocin concentrations no different to non-breeding individuals, showing that expressions of maladaptive maternal behaviour occur during seasons when mothers fail to make the transition from the non-breeding basal, level A, to the breeding basal, level B.

The most probable cause of the failure of some mothers to transition between the non-breeding and breeding basal plasma concentrations of oxytocin is failure of the mother

to bond with and recognise the pup as her own. Oxytocin is crucial for the development of mother-infant bonds (Nagasawa *et al.* 2012) and human mothers with strong bonds with their offspring have higher plasma oxytocin concentrations (Levine *et al.* 2007) and trigger greater peripheral and central oxytocin release when interacting with them than mothers with poor bonds (Strathearn *et al.* 2009). It has been demonstrated in other species with olfactory based offspring recognition systems that there is a limited period of heightened sensitivity in the olfactory bulb for 1-4 hours after parturition modulated by the release of neurotransmitters crucial to olfactory memory such as γ-aminobutyric acid (GABA), to allow mother-offspring bonds to form (Alexander *et. al.* 1986, Lévy *et al.* 1995, Kendrick *et al.* 1992, Lim and Young 2006). If the bond does not form within this window, then there is no other opportunity in that breeding event to rectify this error (sheep, Alexander *et al.* 1986). Oxytocin is only released when interacting with an individual that the mother recognises and shares a positive bond with (Levine *et al.* 2007, Strathearn *et al.* 2009). Therefore without the strong mother-pup bond formed at birth, there can be no oxytocin release through any interactions with it, no transition to the 'breeding basal' plasma level and no correct expression of maternal behaviour. This chain of events can ultimately lead to abandonment of the pup, as mothers with low plasma oxytocin have been found to react to stimuli associated with their infants with fear and disgust (humans, Strathearn *et al.* 2009), driving a mother to reject the infant that she should be caring for. This effect was demonstrated with two of the study mothers which both abandoned their pups in one study year but raised them successfully in the other across the 2010 and 2011 seasons; 'Femsneck' abandoned her pup almost immediately after birth and throughout that breeding season was never observed to interact with it. 'O8' interacted with her pup initially and attempted to nurse it for four days after birth, and then abandoned it. Despite having four days of contact with her pup, O8 had similarly low oxytocin concentrations (3.3pg/ml) to Femsneck (3.7pg/ml), which contrasts greatly to the seasons where they both successfully raised their pups (O8: 9.9pg/ml, Femsneck: 8.2pg/ml).

There is no direct neurological evidence that grey seal mothers experience a similar heightened bonding period immediately after birth as seen in other species using olfaction to recognise their offspring, however, there is behavioural evidence indicating the importance of the time period just after birth. Grey seal mothers that have just given birth make the most physical contact with their pup within the first hour of its life (Burton *et al*. 1975), a comparable window to the period of post-partum sensitivity in

sheep (Alexander *et al.* 1986) and it is widely thought that smell is crucial to mothers trying to identify their pups (Fogden, 1971). Therefore it seems probable that olfaction is playing a role in the recognition system between mothers and pups in grey seals, and that the first hour of the pup's life is important for establishing the mother-pup bond. Immediately after birth there are many distractions that may prevent a mother from staying in close contact with her pup during this crucial period: other breeding adults may attack the pair if they are perceived to have encroached too close to their neighbours (Fogden 1971), and large aggressive gull species, such as the great black backed gull (*Larus marinus*), harass the mother-pup pair to scavenge the placenta (Boyd *et al.* 1962). If the mother chooses to defend the placenta while the gulls attempt to steal it, she can be drawn away from the pup for a considerable period of time, and the pup itself can be predated by the gull pecking at its umbilicus or other vulnerable areas such as the eyes (Boyd *et al.* 1962). With such distractions, a mother can be occupied with aggressive displays towards other conspecifics and gulls immediately after birth, and bonding with the pup may occur too late or not at all.

The nature of the mechanism driving the process of mother-pup bonding in grey seals provides a potential explanation for why mothers who in previous years were successful in raising a pup to weaning may suddenly abandon the current pup, only to have a successful season the following year. The persistence of maladaptive behaviour in a species' and individual's repertoire is interesting given that selection pressure should drive the majority of aspects of an organism's life history to be beneficial to its survival (Darwin 1859). Expressions of maladaptive behaviour can be split based on their frequency, with some forms occurring consistently throughout an individual's life while others occur sporadically and seemingly randomly amongst optimal expressions of the behaviour in question. There are several theories postulating how persistent maladaptive behaviour may be selected for, or not selected against, in a population. Variability in foraging strategies despite consistent food sources is hypothesised to be a way to detect change in the environment and new resources, since an organism that only exploits one nutritional opportunity will never be able to detect new ones no matter how efficient it is (Bernstein, 1984). If a behaviour that appears to be maladaptive in one context of an individual's life is inextricably linked to persistently high rewards in other situations (Sih *et al*. 2004), it has been hypothesised that it is a product of consistent 'personality syndromes' in that animal species (Gosling and John, 1999). One example of this exists in North American fishing spiders (*Dolomedes trition*), where females that are

extremely aggressive and voracious in foraging and feeding contexts achieve larger adult body masses and have increased fecundity but show high frequencies of precopulatory sexual cannibalism (Johnson and Sih 2005). Other persistent maladaptive behaviour may occur due to developmental constraints placed on an individual as it matures, such as the 'abused abuser' cycle of parental neglect or maltreatment carrying across generations (Main and Goldwyn, 1984, Langeland and Dijkstra 1995).
Despite the existence of multiple hypotheses explaining the existence of persistent maladaptive behaviour in an individual, there is currently only one potential explanation for why infrequent maladaptive behaviours occur in individuals that otherwise behave optimally in that context. Extrinsic stochastic events that directly impact on an individual's welfare may render them unable to behave optimally during that season, such as seasonal resource limitations leading to infanticide during that year's breeding event (reviewed in Hrdy 1979) or injuries from a predation event negatively impacting on foraging success during the healing process (McNamara and Houston 2008). None of the mothers in this study appeared to be hindered by such problems, and yet some individuals still were inconsistent in their ability to raise a pup to weaning across the study period. It has been postulated that it is incorrect to assume all behavioural and physical traits are adaptive (Gould and Lewontin 1979, Bernstein 1984), however while not all traits may directly benefit an individual, those that entail an energetic or welfare cost with no payoff should be eliminated from the repertoire of a species. The endocrinological explanation for the failure of the mother-pup bond to form outlined in the above paragraphs provides the first hypothesis to explain the apparently random failure of a behaviour's expression in an individual that has otherwise consistently been successful in that context, and in this case has a direct impact on the individual's reproductive success without any perceivable benefits to either the mother or pup.

5.4.3. Correlating variation in plasma oxytocin concentrations and maternal behaviour in grey seal mothers

The behavioural component for 'awareness of pup' behaviours was positively correlated to plasma oxytocin concentrations in grey seal mothers. Oxytocin is associated with higher numbers of infant checks in human mothers (Feldman *et al.* 2007) and is implicated in primate mothers immediately after birth (Broad *et al*. 2006) and the findings here concur with these existing studies while being the first to directly link infant checks and plasma oxytocin concentrations in a wild mammal species. Both increased activity by the mother to stay close to a pup and high frequencies of pup checks will reduce the likelihood of mother-pup separation during lactation, which is credited for causing starvation, the largest cause of pup mortality on Scottish breeding colonies (Boyd and Campbell 1971, Anderson *et al.* 1979, Baker and Baker 1988).
The distance between mothers and pups is significantly negatively correlated with plasma oxytocin concentrations. This finding also agrees with the behavioural effects of oxytocin in human mothers and fathers, where plasma oxytocin concentrations and maternal proximity are also negatively correlated (Feldman *et al*. 2007) and intranasal administration of oxytocin in fathers modulates their proximity to their own babies (Weisman *et al.* 2013b). In addition sheep breeds with greater amounts of contact with their lambs have higher plasma oxytocin concentrations than breeds which have little contact between the two (Dwyer 2008). Therefore, oxytocin drives mothers to stay close to their pups, and as a result during interactions or physical contact with each other while in close proximity, mothers with strong bonds release more central and peripheral oxytocin (Strathearn *et al.* 2009). As oxytocin is linked to numerous reward and reinforcement pathways in the brain during optimal maternal behaviour expression (Baskerville and Douglas 2010, Atzil *et al.* 2011), release triggered by the pup's presence then subsequently stimulates mothers to remain close to their pups, forming a positive feedback loop to maintain small mother-pup distances.
The two types of maternal behaviour described above were the only two of those measured that were related to oxytocin concentrations in the plasma of maternal grey seals. There are a number of reasons why other behaviours typically associated with oxytocin release showed no relationship to plasma oxytocin concentrations during the dependant period in this species. Some behaviours, such as nursing, will trigger oxytocin release into the bloodstream (see above, section 5.3.2), however this is rapidly

cleared back to basal concentrations once the stimuli (e.g. the pup suckling) stops. Additionally, oxytocin's role in initiating and maintaining different aspects of maternal behaviour varies greatly across the mammalian species currently studied, with strong differences between species such as sheep (Lévy *et al.* 1992), voles (Olazabal and Young 2006), primates (Broad *et al.* 2006), rats and mice (Ferguson *et al.* 2002). While central and peripheral oxytocin concentrations and release do act on behavioural expression, the number and distribution of oxytocin receptors in a variety of brain regions also play a crucial role in this process on a species and individual level (Insel and Shapiro 1992, Francis *et al.* 2000, Gimpl and Fahrenholz 2001, Francis *et al.* 2002, Lim and Young 2006). Therefore not all behaviours controlled by oxytocin release will be correlated to peripheral concentrations as varying transcription rates of receptors in different brain regions also impact on behavioural expression. Despite this the results presented here show that at least two important aspects of maternal behaviour in grey seals are correlated to individual plasma oxytocin concentrations, in line with findings from other mammalian species.

The time period during lactation was a significant explanatory variable in almost all the models for behavioural component data described in this chapter. In late lactation the percentage time spent performing 'pup care' behaviours (nursing and interacting with the pup) increases and time spent resting and the mean mother-pup distance decreases. This is most likely a response to the changing behaviour a pup exhibits throughout the lactation period, as most phocid seal pups increase the amount of time spent nursing, and therefore the time in close proximity to the mother, with age during the dependant period (Boness and Bowen 1996, Renouf and Diemand 1984).

Along with promoting specific components of maternal behaviour and encouraging close proximity between mothers and pups, the plasma oxytocin concentrations found in mothers was positively correlated to those detected in their pups. Therefore high oxytocin concentrations are not only found in mothers that reduce the likelihood of mother-pup separation, but also with mothers that produce pups with high plasma oxytocin concentrations. Patterns of oxytocin expression and the social behaviours they promote have been found to be linked across parents and infants during short term interactions (Weisman *et al.* 2012a) and to transfer across generations in humans (Feldman *et al.* 2010b). As discussed above, neonatal oxytocin exposure has lifelong impacts on an infant's development, the behaviours they express as adults and aspects of reproductive success (Sohlström *et al.* 2000, Olausson *et al.* 2003, Bales *et al.* 2004,

Cushing *et al.* 2005 and Bales *et al.* 2007). Therefore, in grey seal mothers, oxytocin concentrations promote behaviours that keep the mother-pup pair together, which is crucial for the pup's survival during the dependant period, and additionally affects the hormone's expression in her pup, which likely has lifelong consequences for the pup's development, physiology and behaviour.

5.4.4. Correlating variation in plasma oxytocin concentrations and pup behaviour in dependant grey seal pups

The amount of time a pup spends in 'play' behaviours was significantly positively correlated to plasma oxytocin concentrations in the pup, while additionally being affected by the time during lactation and the date in the breeding season the pup was born. In other species, only social play has been associated with rising oxytocin concentrations (Feldman *et al.* 2010b, Weisman *et al.* 2012a) whereas in this study, pup's play behaviour was defined as solitary play without the involvement of the mother or any other conspecific. Why oxytocin is associated with solitary play in this species is difficult to explain, as oxytocin is typically released during behaviours involving another individual. When engaged in 'play' behaviours pups typically do not move great distances, which may assist a mother in locating her pup on the colony. Therefore, pups with high proportions of play behaviours are more likely to stay in one place in early lactation. Higher frequencies of play behaviour could therefore be helping facilitate mother-pup reunions during the early dependant period when mother-pup distances are typically larger than late in the dependant period (see above, 5.4.3 of the discussion), with the resulting contact between the mother-pup pair causing larger peripheral concentrations of oxytocin.

While 'pup activity' behaviours are not correlated with the oxytocin found in a pup, the variables affecting this behavioural component do support the above findings from the maternal models. Pups on average spend greater proportions of their time alert to their surroundings, actively moving around the colony with large distances separating them and their mothers when their mothers have low plasma oxytocin concentrations. Therefore the pups of mothers with low plasma oxytocin concentrations spend more time separated from them, engaged in movement behaviours which may bring them into contact with other, hostile conspecifics on the colony. Other conspecifics and predation events represent a threat to a pup's welfare or survival in grey seals (Boyd *et al.* 1962),

therefore reducing the amount of movement away from the mother is important for maximising its wellbeing. Once again it is oxytocin in the mother that seems to be driving the maintenance of close mother-pup proximity for the benefit of the pup, despite pups having much higher and more variable plasma oxytocin concentrations. Rate of maternal mass loss (kg/day) was the only variable significantly linked to the amount a pup rests and 'interaction with mother' behaviours (suckling and interacting) during the dependant period. It is unsurprising that the rate of maternal mass loss is positively correlated to the amount a pup suckles from its mother, or that pups that rest more have mothers with lower rates of mass loss, as they do not have to expend resources seeking out active pups around the colony. However the relationship between a pup's plasma oxytocin concentration, it's mother's and the mother's rate of mass loss is an important finding given the effects of high maternal expenditure during a single breeding season. Mothers that invest heavily in their pups during one breeding season may increase its likelihood of survival in its first year of life (Hall *et al.* 2001 and 2002) but this negatively impacts on the mother's breeding success in successive seasons (Pomeroy *et al.* 1999). Pups with high plasma oxytocin concentrations have mothers with high plasma oxytocin, and additionally have lower rates of mass loss per day. The hormone therefore seems to be stimulating greater proximity and pup vigilance behaviours in mothers without additional energetic cost to mother grey seals. This allows the expression of maternal behaviour that reduces the likelihood of mother-pup separation, while not increasing the rates of maternal mass loss. Oxytocin release in human mothers has been linked to optimal expression of maternal behaviour as oppose to 'high risk' parenting (Atzil *et al.* 2011), and the hormone may fulfil the same function in grey seal mothers, rewarding the form of maternal behaviour expression that provides the greatest benefit to the pup at the least cost to the mother, but avoiding extremes of the behavioural spectrum, ranging from negligence or complete lack of maternal care to over-investing in one pup during one season. Therefore while oxytocin seems to have no impact on the majority of behaviour a pup displays while with its mother, the concentrations present across the two individuals in the mother-pup pair have a complex relationship that promotes a strong bond between the two. This is then associated with minimal periods of separation without additional energetic investment from the mother, maximising a pup's likelihood of reaching weaning age without additional cost to the mother during this season and future reproductive events.

5.5. Conclusions

This study provides the first documentation of plasma oxytocin concentrations in a wild mammal species, and defined the basal plasma oxytocin concentrations of three species of phocid seal, of both sexes. In grey seals, data were generated across multiple life history stages. The plasma concentrations detected show that oxytocin follows the pattern of expression described by the Challenge Hypothesis in female grey seals, with mothers transitioning to a 'breeding basal' plasma oxytocin concentration when with their pups. Mothers that fail to make this transition have plasma oxytocin concentrations comparable to non-breeding individuals and perform behaviours that are detrimental to the pup's welfare and its likelihood of survival to weaning. Finally the plasma oxytocin concentrations present in grey seal mothers and dependant pups was successfully measured. Pups had concentrations that were at least three times higher than their mothers, and this high concentration in pups dropped after weaning. Mother and pup concentrations were positively correlated, and mothers with higher oxytocin concentrations engaged in more pup checking behaviours, maintained smaller distances between the two while on the colony and had pups that actively moved around the colony less. All these behaviours reduce the likelihood of mother/pup separations occurring, which is cited as the ultimate cause of the biggest cause of pup mortality on a breeding colony (starvation). High pup oxytocin was additionally associated with mothers with high plasma oxytocin and low rates of daily maternal mass loss, indicating that the hormone promotes optimal forms of maternal behaviour expression that increase the likelihood of success in that reproductive event without additional cost to the mother.

Chapter 6. Manipulating Oxytocin

6.1. Introduction

There are many different study designs that can be employed to investigate whether two variables are dependant on one another. Correlational research, as used in Chapter 5 of this thesis, is an excellent way to explore the natural relationships existing between variables and provides vital information to guide future research questions and experiments. However, only experimental research can test and establish which variables are the causes of behavioural or physiological change and which are the subsequent effects. Experimental research involves the manipulation of a variable while observing any effects on the hypothetical dependant variable, and is considered the best way to obtain accurate data on systems relying on hormonal or chemical influences on individual physiology or behaviour (Walker 2005). The North Rona and Isle of May grey seal colonies offer an excellent opportunity to record natural behavioural frequencies and to take plasma samples alongside these observations. However in order to directly link oxytocin concentrations in the plasma of grey seals to the behaviour they express, manipulation experiments must be performed to deduce any relationship that may exist between the two.

Oxytocin manipulations have been successfully carried out on a variety of mammalian species to influence their behaviour. Rats (Popik *et al.* 1992, Dluzen *et al.* 1998), mice (McCarthy 1990, Ferguson *et al.* 2001), voles (Cho *et al.* 1999, Bales *et al.* 2007, Grippo *et al.* 2009), meerkats (Madden and Clutton-Brock 2011), sheep (Lévy *et al.* 1992 and 1995, Da Costa *et al.* 1996), marmosets (Smith *et al.* 2010) and humans (Heinrichs *et al.* 2003, Domes *et al.* 2007, Hollander *et al.* 2007, Ditzen *et al.* 2009, Hurlemann *et al.* 2010) have all undergone oxytocin manipulation trials using a variety of delivery methodologies and doses, resulting in significant changes in their behavioural expression when compared to controls. Therefore it should be possible to develop a protocol for manipulating oxytocin concentrations in grey seals to allow direct testing of the hormones effects on an individual's behavioural expression. Determining which methodology to use to administer the oxytocin dose is the first challenge to overcome in this process. The routes favoured by researchers currently are those that deliver the oxytocin dose straight to central brain regions. This is

achieved either by injection directly into target brain structures to trigger behaviours associated with that brain region (Lévy *et al.* 1992, Da Costa *et al.* 1996, Dluzen *et al.* 1998, Cho *et al.* 1999, Ferguson *et al.* 2001) or via intranasal sprays (Heinrichs *et al.* 2003, Kosfeld *et al.* 2005, Ditzen *et al.* 2009, Rimmele *et al.* 2009, Smith *et al.* 2010). However neither of these methodologies would be applicable to use on wild grey seals, central injections being too invasive and intranasal sprays being impractical in a species that can close its nostrils and hold its breathe for at least 25 minutes (Thompson and Fedak 1993). Manipulation experiments have been conducted using peripheral administration of oxytocin, utilising oxytocin injections subcutaneously (McCarthy 1990, Popik *et al.* 1992, Grippo *et al.* 2009), IV (Hollander *et al.* 2007), IM (Madden and Clutton-Brock 2011) and intraperitoneally (Bales *et al.* 2007) to alter behavioural expression successfully in trial individuals. However such studies are in the minority, as oxytocin is widely reported to be unable to pass through the blood brain barrier (BBB) in physiologically significant amounts (Ermisch *et al.* 1985a, Kang and Park 2000, Churchland and Winkielman 2012). Oxytocin additionally has a rapid plasma clearance rate from peripheral circulation in all mammalian systems currently studied (rats; Lundin *et al.* 1993 and Morrin *et al.* 2008, goats (*Capra aegagrus hircus*); Homeida and Cooke 1984, baboons; Dawood *et al.* 1979 and humans; Thornton *et al.* 1990). Therefore any peripherally administered manipulation of the hormone must be calculated so that it persists across the required timescale for any behavioural effects to become evident, while also taking into account any variation the route of administration (intravenous (IV) or intramuscular (IM)) may have on the plasma clearance rate.

Despite studies asserting that the BBB has poor permeability to oxytocin, the successful manipulation of behaviour via peripheral injections provides compelling evidence that central brain structures are reached using this methodology (Viero *et al.* 2010). The precise permeability of the BBB to oxytocin has additionally been calculated in one study, demonstration that 0.002% of a subcutaneously administered oxytocin dose is detectable in the cerebrospinal fluid (CSF) of rats 10 minutes after injection (Mens *et al.* 1983). Therefore if the administered dose is high enough for long enough post injection, physiologically significant amounts of plasma oxytocin may penetrate the BBB and bind to central oxytocin receptors, triggering behavioural responses. This chapter therefore looks to develop doses of oxytocin for phocid seals

that are suitable for IV and IM administration to be used in hour long behavioural trials.

The objectives of this chapter were:

1. To develop a suitable dose of oxytocin to administer intramuscularly to mother grey seals to elevate plasma oxytocin concentrations for one hour duration behavioural manipulation trials.
2. To develop a suitable dose of oxytocin to administer intravenously to juvenile or newly weaned grey seals to elevate plasma oxytocin concentrations for one hour duration behavioural manipulation trials.
3. Determine the plasma clearance rates of injected oxytocin administered by different routes in both adult mothers and newly weaned grey seals.
4. To explore whether the developed manipulations penetrate central brain regions and trigger changes in behavioural expression during the one hour behavioural manipulation trials.

6.2. Methods

6.2.1. Determination of a suitable manipulation dose for adult female grey seals and calculating the plasma clearance rate

Study Animals

Seven adult female grey seals with dependant pups (mothers) where utilised to trial an IV dose of oxytocin on the North Rona breeding colony from the 3rd October 2010 until the 31st October 2010. One female was utilised for two clearance trials, one during the 'early' sampling opportunity and the second during the 'late' sampling opportunity (see Chapter 2. for definitions).

Seven mothers from the Isle of May colony were utilised to test IM delivery of the developed oxytocin dose from the 19th November 2011 until the 1st December 2011. One female was utilised for two clearance trials, one during the 'early' sampling opportunity and the second during the 'late' sampling opportunity (see Chapter 2. for definitions)

Dose Trials and Plasma Sampling

Dose development trials were conducted to develop an appropriate IM dose for manipulating oxytocin concentrations in grey seal mothers. The objective of the manipulation was to elevate successfully plasma oxytocin concentrations in an individual for one hour (the duration of behavioural trials in order to test the effects of such hormone changes on behaviour expression). One IM dose of oxytocin has been used successfully to cause behavioural changes in free ranging wild mammals (meerkats, 0.18µg/ml/kg oxytocin, Madden and Clutton-Brock 2011). This is a suitable dose for small domestic mammals such as cats and dogs (Intervet Ltd, Ireland) but doses for large mammals, such as cows, are larger and therefore this was potentially too small for a large mammal such as a phocid seal. Therefore dose had to be adjusted to a volume that could realistically be injected while still delivering a dose high enough to affect an individual's behaviour. The proposed dose was adapted from the IM dose given for large domestic mammals such as cows and horses, giving an IM injection of 4ml of 180µg/ml for each individual. As the appropriate IV dose of oxytocin is a quarter of the IM dose (Intervet Ltd) and as a 1ml IV injection of oxytocin is know to stimulate milk let down in this species (Pomeroy pers. comm), an IV injection of 1ml of 180µg/ml of Intervet oxytocin was used in the first dose trial. As no data exists on the plasma clearance properties of oxytocin in any phocid species, using a dose which is know to trigger physiological changes was a good starting point for this study.

Oxytocin has a rapid clearance rate in plasma, and is reported to have a terminal half-life during the elimination phase of approximately 20 minutes in goats (Homeida and Cooke 1984) and rats (Lundin *et al.* 1993). As the dose given to elicit a physiological response in mothers (180µg/ml, approximately 857.1ng/kg) was less than half that given in the goat study (2000ng/kg, Homeida and Cooke 1984), and as no data on phocid plasma clearance rates for this hormone exists, the time until plasma concentrations returned to basal was unknown. Therefore the duration of the serial sampling was set to 20 minutes, reflecting the duration of serial plasma sampling used in other low dose oxytocin clearance trials in rats (Morin *et al.* 2008). All eight mothers used for this dose trial were captured and chemically immobilised as described in Chapter 2. A plasma sample was taken immediately before the clearance trial began from the extradural vein to establish a basal plasma oxytocin concentration for each mother used in the study. All mothers then received a 1ml 180µg/ml IV

injection of oxytocin into the extradural vein, and subsequently had a series of plasma samples taken from the same site to allow detection of the plasma clearance of the hormone. Plasma samples were taken at 1, 2, 3, 4, 5, 10, 15 and 20 minute intervals from the oxytocin injection for all eight females.

Based on the results of this manipulation, trials testing the IM administration of the dose were conducted. To convert the IV dose into an IM dose, the IV dose was multiplied by four as per the instructions given with the oxytocin drug (Intervet UK Ltd), giving a dose of 4ml of 180µg/ml for the IM manipulation. All eight mothers used for this dose trial were captured and chemically immobilised as described in Chapter 2. All doses were guaranteed to be injected into the muscle and not the blubber layer. A plasma sample was taken immediately before the clearance trial began from the extradural vein to establish a basal plasma oxytocin concentration for each mother used in the study. All mothers then received a 4ml 180µg/ml IM injection of oxytocin into the flank, and subsequently had a series of plasma samples taken from the extradural vein to allow detection of the plasma clearance rate of the hormone. Plasma samples were taken at 1, 2, 3, 4, 5, 10, 15, 20, 40 and 60 minute intervals from the oxytocin injection for all eight females.

Plasma Analysis

All plasma samples were stored and extracted using the protocols described in Chapter 2 with the following changes to permit analysis of samples containing very high oxytocin concentrations. All samples from the IV manipulation trials were extracted from 3ml of plasma. All samples taken one minute after the oxytocin injection were run on the ELISA plate at x100 and x1000 dilutions. All other samples were run undiluted and at x100 dilutions. For samples from the IM manipulation trials, all samples were extracted from 1ml of plasma and run on the ELISA plates undiluted and at x5 dilutions. These extraction volumes and dilution factors meant that all samples fell within the detection range of the ELISA kit used. Inter-assay coefficient of variance across the 15 plates used in this study was 7.9%.

Statistical Analysis

All analysis was performed using the statistical package R 2.15.0 (R Development Core Team 2012). GAMs (Wood 2006a) were used to generate the clearance curves for both the IV and IM doses. Both models were fitted with a gamma error

distribution and used one random effect (minutes post oxytocin injection). As this variable was not linear it was fitted as a smooth function (Wood, 2006b) with smoothing parameters set by maximum likelihood (Wood, 2011). Model selection was carried out by using AIC to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models (Richards 2005). The plasma clearance rate (CL) for both IV and IM injections of oxytocin were calculated using the following equation:

$$CL = D/AUC$$

Where AUC is the area under the curve generated in the GAMs described above describing the relationship between plasma oxytocin concentrations and time post injection and D is the initial bolus of oxytocin injected in picograms (pg).
Plasma oxytocin concentrations from samples taken just before the clearance trials (basal) and samples one hour after the injection were compared by a Mann Whitney U test. A non parametric test was used as the data was not normally distributed when submitted to a Shapiro Wilk test ($p=0.005$) and variance across the two groups was not equal when examined with a Levene test ($p=0.02$).

6.2.2. Determination of a suitable manipulation dose for juvenile and newly weaned seals and calculating the plasma clearance rate.

Study Animals

Two captive harbour seals held at the SMRU were initially utilised to develop the IV dose for young phocids. Dose trials were conducted from the 7th June 2011 until the 13th September 2011. Individuals V (two year old male harbour seal) and W (one year old female harbour seal) where used for this study.
Individual V was additionally used for testing the plasma clearance rate of one of the developed doses on the 22nd July 2011. Five newly weaned grey seal pups were also subsequently used to test the plasma clearance rate of two of the developed doses on the Isle of May on the 2nd December 2012.

Dose Trials and Plasma Sampling

Dose development trials were conducted to develop an appropriate IV dose for manipulating oxytocin concentrations in juvenile or newly weaned phocid seals. The objective of the manipulation was to elevate successfully plasma oxytocin concentrations in an individual for one hour (the duration of behavioural trials in order to test the effects of such hormone changes on behaviour expression). The first dose was developed using two captive harbour seals at SMRU based on the doses given for domestic mammals and used successfully to cause behavioural changes in free ranging wild meerkats (Madden and Clutton-Brock 2011). To convert an IM dose to an IV dose, a quarter of the IM dose should be used, giving 0.0045µg/ml/kg IV (Intervet UK Ltd). As the blood volume of a phocid seal can be estimated as a percentage of its body mass (Castellini and Somero 1981 and reviewed in Kooyman 1989), the oxytocin spike in the plasma of an individual could be calculated (Table 6-1) to determine how close the concentration was to naturally observed rises in plasma oxytocin, as described in Chapter 5. As these spikes would have been more than a thousand times higher than any naturally occurring oxytocin peak (30pg/ml as described in Chapter 5.), the dose was reduced to a fifth of that used in the meerkat experiment. This dose of 0.0009µg/ml/kg still generated a spike approximately two hundred times higher than any recorded natural peak in oxytocin, but was used for the first trial dose as it was uncertain how high the dose needed to be to successfully cause behavioural changes.

To test whether the 0.0009µg/ml/kg dose could successfully raise plasma oxytocin concentrations for one hour, both V and W received a randomised IV injection of either saline or oxytocin into the extradural vein. Whichever treatment they did not receive during the first trial they received during the second trial a day later. The individuals were not anesthetised during the manipulation or the one hour period after the injection, but were manually restrained for the injection and then placed in a circular holding pen alone (5m diameter) for the hour until sampling. Plasma samples were taken one hour from the oxytocin or saline injection and were taken from the extradural vein using the methodology outlined in Chapter 2.

Table 6-1. Morphometrics and dose calculations for V and W using the first two IV doses considered for trial, one based on a prior successful manipulation experiment (0.0045µg/ml/kg) and the other a quarter of this dose to more closely mimic natural plasma oxytocin peaks (0.0009µg/ml/kg).

ID	Mass (kg)	Blood Volume (litres)	Dose used (µg/ml/kg)	Hypothetical plasma peak generated (pg/ml)
V	29.8	3.9	0.0045	34615.4
W	21.4	2.8	0.0045	32142.9
V	29.8	3.9	0.0009	6923.1
W	21.4	2.8	0.0009	6428.6

Based on the results of these manipulations, trials testing the lower IV doses were conducted to attempt to match the plasma spike generated to those naturally observed in wild, free ranging individuals while still elevating concentrations for the entire hour of the proposed behavioural trials. Individual V was used for four subsequent dose trials, injecting approximately three quarters (0.00067µg/ml/kg), half (0.00045µg/ml/kg), one quarter (0.00022µg/ml/kg) and on eighth (0.00011µg/ml/kg) of the first 0.0009µg/ml/kg dose trialled (Table 6-2). The same trial methodology as outlined above in the initial dose development trials for juvenile individuals was used in these trials, with one amendment as saline controls were not conducted alongside the oxytocin manipulations. The most appropriate dose for use in all future behavioural manipulation trials (0.00045µg/ml/kg) was then subsequently tested on individual W using the same trial methodology but without a saline control (Table 6-2).

Table 6-2. Morphometrics and dose calculations for V and W using the four subsequent IV doses considered for trial, based on the results of the first dose trial.

ID	Mass (kg)	Blood Volume (litres)	Dose used (µg/ml/kg)	Hypothetical plasma peak generated (pg/ml)
V	29.8	3.9	0.00067	5192.3
V	29.8	3.9	0.00045	3461.5
V	29.8	3.9	0.00022	1730.8
V	29.8	3.9	0.00011	946
W	21.4	2.8	0.00045	3214.3

To investigate the clearance properties of the dose that was selected for use in the behavioural trials and of the dose that was not successful in elevating plasma oxytocin concentrations for more than an hour, a set of serial sampling experiments were conducted using individual V and five newly weaned grey seals. All individuals were captured and restrained using the methodology described in Chapter 2. and chemically immobilised using an IV injection of zoletil into the extradural vein. A plasma sample for calculation of basal oxytocin concentrations in each individual was taken, and then each study animal was given either a 0.00011µg/ml/kg or 0.00045µg/ml/kg IV injection of oxytocin. Individual V and two of the grey seals were given the 0.00011µg/ml/kg dose and the remaining three grey seals were given 0.00045µg/ml/kg. Plasma samples were then drawn from the extradural vein at 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50 and 60 minutes post injection.

Plasma Analysis

All plasma samples were stored and extracted using the protocols described in Chapter 2. with the following changes to permit analysis of samples containing very high oxytocin concentrations. All samples taken after the 0.0009µg/ml/kg ml/kg oxytocin manipulations were extracted from 1.5ml of plasma for analysis. All samples taken post saline manipulation and post all other oxytocin doses were extracted from 3ml of plasma. These extraction volumes and dilution factors meant that all samples fell within the detection range of the ELISA kit used with the exception of one, which was unexpectedly higher than the top of the detection curve (the sample for the

0.0036ml/kg dose). Inter-assay coefficient of variance across the 5 plates used in this study was 2%.

All samples from the clearance trial with individual V and the 0.00011μg/ml/kg dose were extracted from 3ml of plasma. All samples taken 1-4 minutes from the oxytocin injection were run on the ELISA plate undiluted and at x100 dilutions. All other samples were run undiluted. For samples from the two other IV manipulation trials using 0.00011μg/ml/kg and newly weaned grey seals, all samples were extracted from 1ml of plasma. Samples taken 1-4 minutes from the oxytocin injection were run on the ELISA plates undiluted and at x5 dilutions, while samples taken from 5-60 minutes were run undiluted. For samples from the three newly weaned grey seals given the 0.00045μg/ml/kg dose, all samples were extracted from 1ml of plasma. Samples taken 1-3 minutes from the oxytocin injection were run on the ELISA plates at x5 and x10 dilutions, while samples taken from 4-60 minutes were run undiluted and at x5 dilutions. Inter-assay coefficient of variance across the 6 plates used in this study was 7%.

Statistical Analysis

All analysis was performed using the statistical package R 2.15.0 (R Development Core Team 2012). GAMs (Wood 2006a) were used to generate the plasma clearance curves for both IV doses. Both models were fitted with a gamma error distribution and used one random effect (minutes post oxytocin injection). As this variable was not linear it was fitted as a smooth function (Wood, 2006b) with smoothing parameters set by maximum likelihood (Wood, 2011). Model selection was carried out by using the AIC and to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models.

The plasma clearance rate (CL) for both IV doses was calculated using the same equation as described above using the AUC from the GAM models describing their plasma clearance rates.

Plasma oxytocin concentrations from samples taken just before the clearance trials (basal), samples one hour after the injection with 0.00045μg/ml/kg and 0.00011μg/ml/kg were compared by a one way ANOVA. The data was analysed after a natural logarithmic transformation as the original data was not normally distributed when submitted to a Shapiro Wilk test ($p=0.002$).

6.2.3. Exploration of potential of doses to affect central brain regions

Statistical Analysis

All analysis was performed using the statistical package R 2.15.0 (R Development Core Team 2012). GLM were used to analyse the variables that could affect the plasma clearance rate of the three different manipulation doses and methodologies (the IM 4ml 180µg/ml injection, the IV 0.00045µg/ml/kg dose and the IV 0.00011µg/ml/kg dose). Models were fitted with a gamma error distribution with the dose type (4ml 180µg/ml, 0.00045µg/ml/kg or 0.00011µg/ml/kg), the route of dose administration (IV or IM), the sex of the individuals used in the trial and the minutes since the oxytocin manipulation was given fitted as fixed effect variables. Model selection was carried out by using the AIC to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models (Table 6-3).

Table 6-3. Model variables and selection process for the GLM examining the three oxytocin doses and the variables that affect their clearance.

Model type	Fixed Effects	Kept in final model?	Levels
GLM	Dose	Yes	4ml 180µg/ml, 0.00045µg/ml/kg or 0.00011µg/ml/kg
	Route	Yes	IV, IM
	Sex	Yes	Male, Female
	Time post injection	Yes	Continuous (in minutes)

6.3. Results

6.3.1. Determination of a suitable manipulation dose for adult female grey seals and calculating the plasma clearance rate.

IV dose trial

The plasma clearance curve for the 1ml IV dose of oxytocin was successfully detected from the serial plasma samples collected during the first 20 minutes from injection

(Figure 6-1). Two distinct phases were apparent in the curve, a distribution phase where the plasma oxytocin concentration halved every minute from 1-5 minutes post injection and an elimination phase from minute 5 onwards, where the plasma clearance rate slowed and plasma concentrations took ~15 minutes to halve (Table 6-4). The plasma clearance rate of this dose was 0.025 L/min/kg.

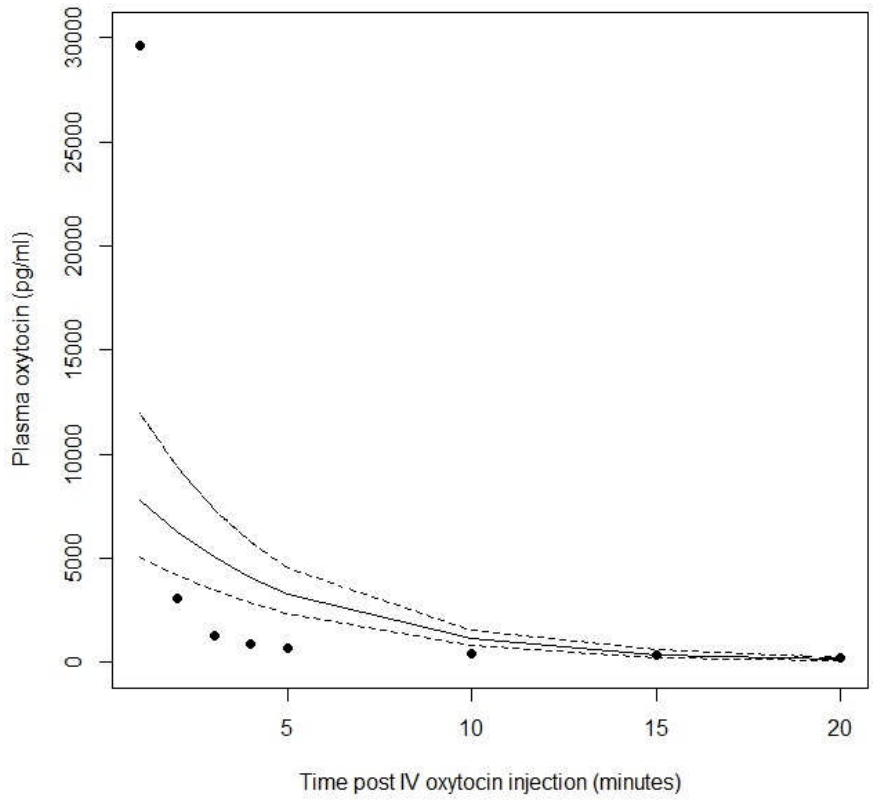


Figure 6-1. Mean plasma oxytocin concentrations detected in adult female grey seals at eight time points post IV injection with 1ml of 180µg/ml Intervet Ltd oxytocin and the clearance curve generated from them with confidence intervals (dashed).

IM dose trial

The plasma clearance curve for the 4ml 180µg/ml IM dose of oxytocin was successfully detected from the serial plasma samples collected during the first 60 minutes from injection (Figure 6-2). Plasma oxytocin concentrations took between 2-4 minutes to peak after the injection and after peaking, concentrations halved every minute until 5 minutes post injection. Beyond the first five minutes, the plasma

clearance rate was much slower, taking approximately 30 minutes to halve (Table 6-4). The plasma clearance rate of this dose was 0.22 L/min/kg.

The IM dose successfully raised the plasma oxytocin concentrations above basal concentrations for an hour. Plasma concentrations from samples at 60 minutes post injections were all significantly higher than the basal concentrations detected in those individuals prior to the trial ($W(16)=0$, $p<0.001$) (Figure 6-3).

Table 6-4. Mean values with standard errors for the plasma oxytocin concentration detected in mothers post two different types of oxytocin manipulation.

Time post injection (minutes)	1ml 180µg/ml IV dose (pg/ml)	4ml 180µg/ml IM dose (pg/ml)
Basal (pre-injection)	13.1 ±1.2	11.6 ±1.4
1	29654.5 ±8052.3	344.2 ±183.7
2	3059.2 ±1429	717.9 ±406
3	1283.3 ±202.3	1206.6 ±602.9
4	863.2 ±185.7	1139.6 ±523.6
5	663.5 ±117.7	619.6 ±174.8
10	450.9 ±79.2	479.1 ±129.7
15	374.5 ±56.7	493.6 ±128.3
20	220.8 ±48	302.8 ±90
40	X	235.7 ±25
60	X	224.5 ±29.4

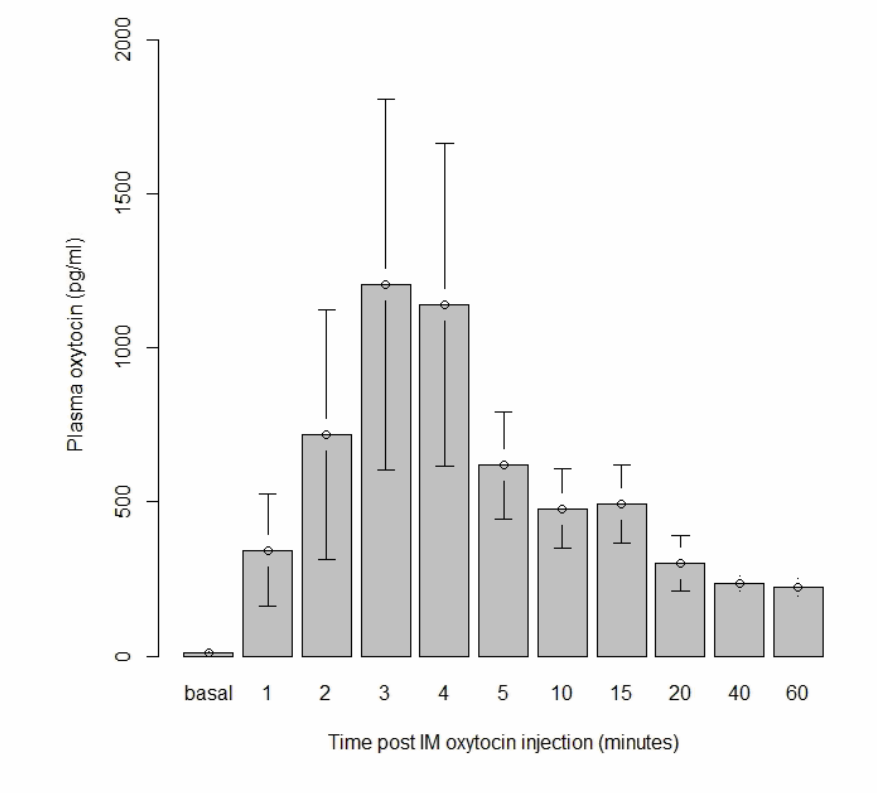


Figure 6-2. Mean plasma oxytocin concentrations with standard error bars from adult female grey seals detected at 10 time points post IM injection with 4ml 180µg/ml of Intervet Ltd oxytocin.

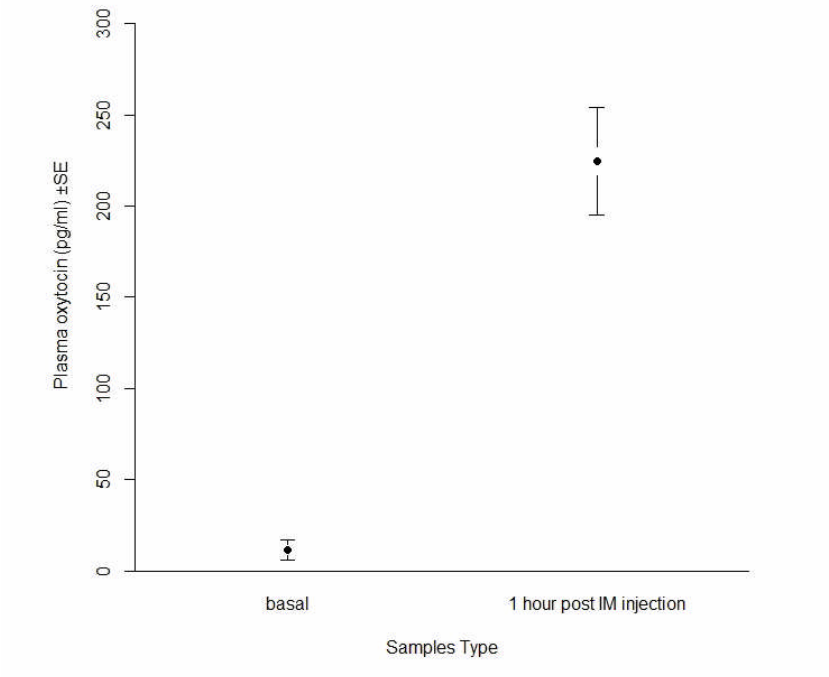


Figure 6-3. Mean basal plasma oxytocin concentrations and those detected at 1 hour post IM injection with 4ml 180µg/ml of Intervet Ltd oxytocin from adult female grey seals with standard error bars.

6.3.2. Determination of a suitable manipulation dose for juvenile and newly weaned seals and calculating the plasma clearance rate.

IV dose development

The first dose of 0.0009µg/ml/kg trialled with individuals W and V was successful in elevating plasma oxytocin concentrations above basal (6.1 ±0.55 pg/ml, see Chapter 5.) for an hour after the injection (18 ±2.9pg/ml) when compared to a saline control (5.9 ±0.45pg/ml) (Figure 6-4). Of the four other doses trialled subsequently, one could not be detected using the ELISA (the 0.00067µg/ml/kg dose) and of the remaining three doses all but one was successful in elevating plasma oxytocin concentrations above basal levels for one hour (the 0.00011µg/ml/kg dose, Figure 6-5 and Table 6-4). The 0.00045µg/ml/kg dose was then selected as the dose to use for all behavioural manipulation trials on young individuals (see Chapter 7.), and it successfully elevated the plasma oxytocin concentration of individual W for one hour in the last dose development trial (Table 6-5).

Table 6-5. Plasma oxytocin concentration detected in individuals W and V one hour after different types of oxytocin manipulation.

Dose given (µg/ml/kg)	Plasma Oxytocin Concentrations 1 hour post injection (pg/ml)	
	W (2 trials)	V (5 trials)
0.0009	20.9	15.1
0.00067	X	X
0.00045	16.1	25.3
0.00022	X	12.6
0.00011	X	3

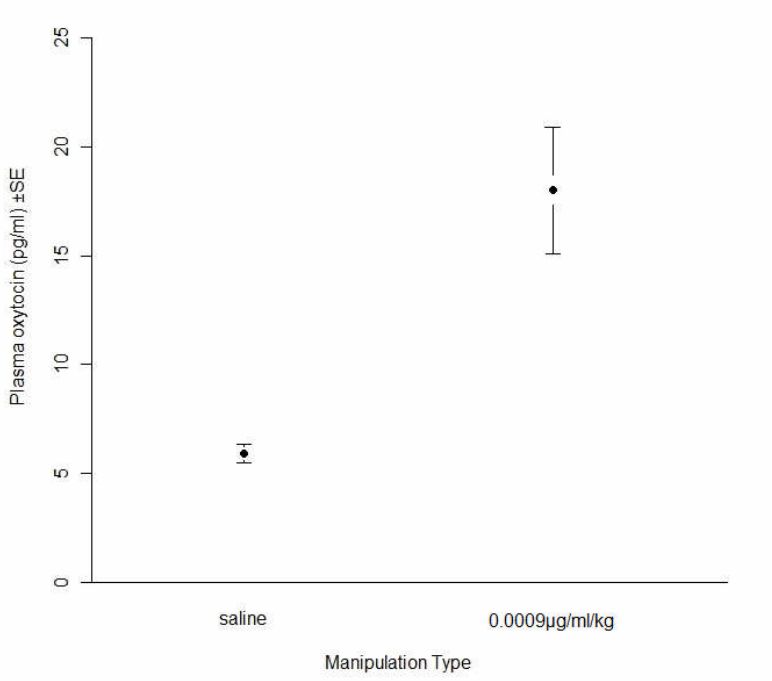


Figure 6-4. Mean plasma oxytocin concentrations detected in individuals W and V one hour after an IV injection of either saline or 0.0009µg/ml/kg dose of oxytocin with standard error bars.

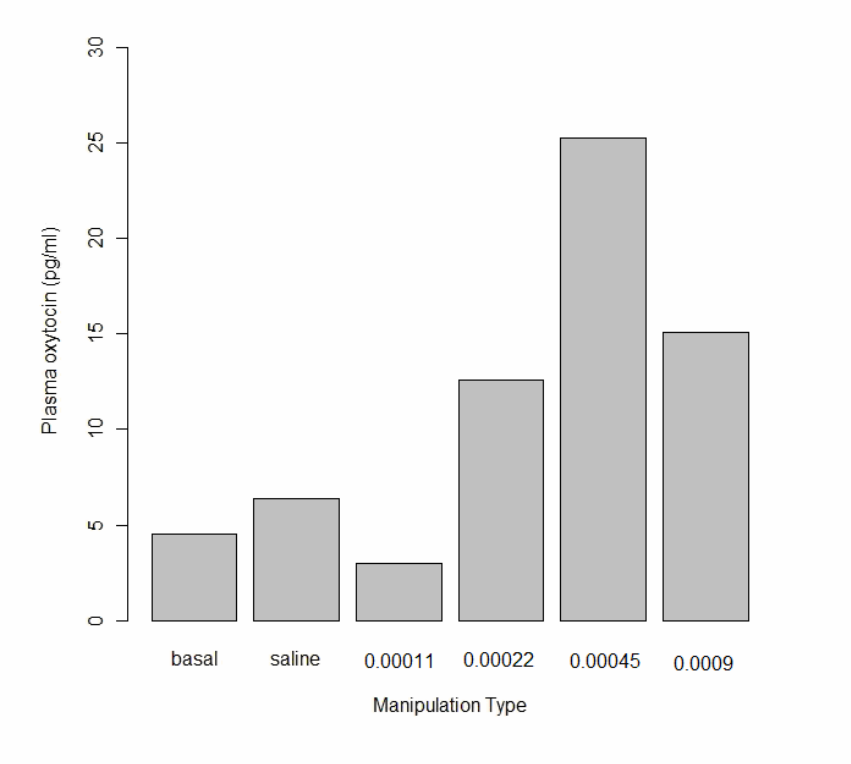


Figure 6-5. Plasma oxytocin concentrations detected in individual V under basal conditions and one hour after an IV injection of either saline or four different doses of oxytocin in µg/ml/kg.

IV dose trial

The plasma clearance curves for the two different IV doses of oxytocin (0.00045μg/ml/kg and 0.00011μg/ml/kg) were successfully detected from the serial plasma samples collected during the hour post injection (Figure 6-6 and 6-7). Similar to the plasma clearance curves from the IV and IM manipulations in mothers described above, the 0.00045μg/ml/kg dose generated a curve with two distinct distribution and elimination phases. During the distribution phase the plasma oxytocin concentration rapidly fell to a quarter of its value during the 1-5 minutes post injection. From the 10 minute sample onwards the plasma clearance rate slowed with the plasma concentration halving approximately every 20 minutes (Figure 6-6 and Table 6-6). The plasma clearance curve for the 0.00011μg/ml/kg dose was different to the other curves previously described, as within the first five minutes plasma oxytocin concentrations fell to $1/15^{th}$ of the original peak, and then halving approximately every 15 minutes until concentrations returned to basal levels at 1 hour post injection (Figure 6-7 and Table 6-6). The plasma clearance rate of the 0.00045μg/ml/kg dose was 0.026 L/min/kg and for the 0.00011μg/ml/kg dose was 0.022L/min/kg.

A one way ANOVA with a Tukey honest significant differences post-hoc test showed the 0.00045μg/ml/kg IV dose successfully raised plasma oxytocin concentrations above basal concentrations for an hour, while the 0.00011μg/ml/kg IV dose did not ($F_{(2,11)}$=17.2, $p<0.001$). Plasma concentrations from samples at 60 minutes post injection using the 0.00045μg/ml/kg dose (52.3 ±14.9pg/ml) were all significantly higher than the basal concentrations (10.5 ±3pg/ml, $p<0.001$) and concentrations 60 minute post injection from the 0.00011μg/ml/kg dose trial (11.6 ±3.3pg/ml, $p=0.003$). There was no difference between samples taken 60 minute post 0.00011μg/ml/kg manipulation and basal plasma concentrations of oxytocin ($p=0.9$) (Figure 6-8).

Table 6-6. Mean values with standard errors for the plasma oxytocin concentration detected in individual V and five newly weaned grey seal pups post two different types of oxytocin manipulation.

Time post injection (minutes)	0.00045µg/ml/kg IV dose (pg/ml)	0.00011µg/ml/kg IV dose (pg/ml)
Basal (pre-injection)	10.5 ±1.2	9.9 ±1
1	2186.3 ±1098.7	1492.8 ±690.9
2	997.1 ±273	452.3 ±135.6
3	986.5±475.3	183.9 ±36.5
4	436.9 ±139.5	133.4 ±33.4
5	495.4±170	100.5 ±21.9
10	180 ±50.1	71.1 ±30.5
15	175.3 ±15.8	61.2 ±16.1
20	132.5 ±31.2	45.4 ±13.6
30	99.6 ±20.8	29.8 ±11.6
40	120.1 ±25.1	25.5 ±7.6
50	85.4 ±8.6	20.8 ±5.9
60	54.2 ±10.7	13.8 ±5.5

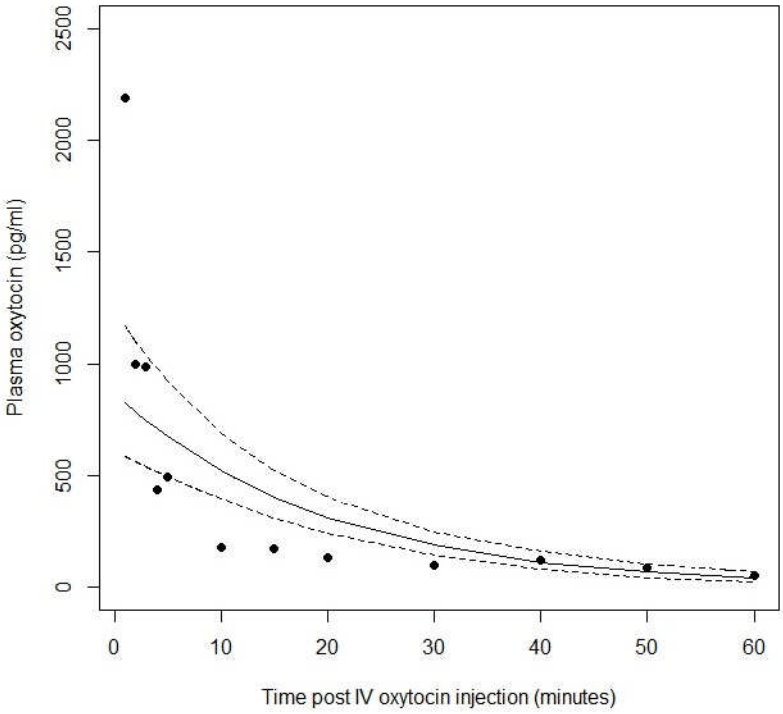


Figure 6-6. Mean plasma oxytocin concentrations from newly weaned grey seals detected at twelve time points post IV injection with 0.00045µg/ml/kg oxytocin and the plasma clearance curve generated from them with confidence intervals (dashed).

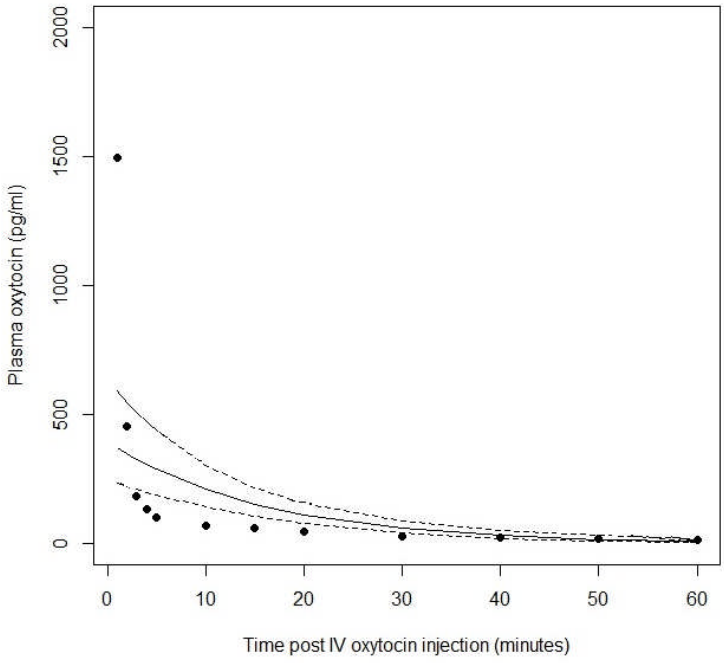


Figure 6-7. Mean plasma oxytocin concentrations from newly weaned grey seals and V detected at twelve time points post IV injection with 0.00011µg/ml/kg oxytocin and the plasma clearance curve generated from them with confidence intervals (dashed).

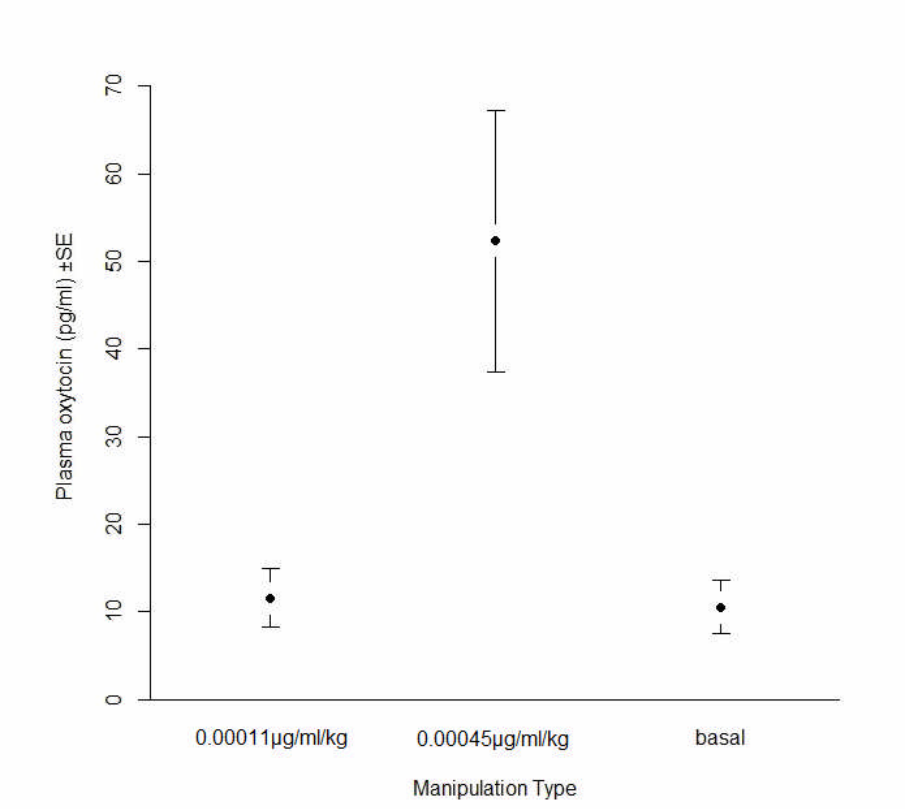


Figure 6-8. Mean plasma oxytocin concentrations detected in individuals during the two IV manipulation trials, showing basal (pre-injection) concentrations, one hour after IV injection of 0.00045µg/ml/kg dose of oxytocin and one hour after IV injection of 0.00011µg/ml/kg dose of oxytocin with standard error bars.

6.3.3. Exploration of potential of doses to affect central brain regions.

Of the variables tested, the GLM model showed that the dose given ($F_{(4, 141)} = -2.9$, $p=0.004$) and time from injection (($F_{(4, 141)} = -6.9$, $p<0.001$) significantly affected the oxytocin detected in samples (Table 6-7). The route of injection (IV or IM) or the sex of the individual did not significantly affect plasma oxytocin concentrations (see appendix X). There was no significant difference between samples generated using the IM 4ml 180µg/ml injection and the IV 0.00045µg/ml/kg dose ($p=0.4$). However there were significant differences between these two doses and samples generated using the IV 0.00011µg/ml/kg dose ($p=0.004$).

Table 6-7. Significant fixed effect variables from the GLM concerned with variables effecting the plasma oxytocin concentration of samples taken during three of the clearance trials (using the IM 4ml 180µg/ml injection, the IV 0.00045µg/ml/kg dose and the IV 0.00011µg/ml/kg dose), their standard errors, estimates and p values.

Dependant variable/explanatory variable	Estimate	Standard Error	P value
Plasma Oxytocin concentration (pg/ml) – Dose type (0.00045µg/ml/kg)	-0.25	0.29	0.39
Plasma Oxytocin concentration (pg/ml) – Dose type (0.00011µg/ml/kg)	-1.04	0.35	0.004
Plasma Oxytocin concentration (pg/ml) – Time since injection (minutes)	-0.04	0.006	<0.001

6.4. Discussion

6.4.1. Determination of a suitable manipulation dose for adult female grey seals and calculating the plasma clearance rate.

The 4ml 180µg/ml dose and IM delivery route was successful in significantly raising the plasma oxytocin concentrations of adult female grey seals for one hour. This methodology could therefore be used in subsequent experiments on free ranging females with dependant pups (Chapter 7.). The dose and administration route allow elevation of plasma oxytocin concentrations for the entire duration of the 1 hour trial period without needing to chemically immobilise the individual. As the degree of immobilisation, the time taken to capture and the time to recover from immobilisation vary greatly between females, darting study individuals to allow administration of an IV dose would not have been a viable trial protocol.

Plasma clearance rates for both the IV and IM oxytocin injections were successfully calculated. The plasma clearance rate of the 1ml IV injection (containing approximately 1107.7ng/kg) of oxytocin in grey seals is comparable to published data on the plasma clearance rate of a similar IV dose given to rats (0.027 L/min/kg for a 1000ng/kg dose, Morin *et al.* 2008). There are no published figures for the plasma clearance rate of oxytocin manipulations given IM, however the higher plasma clearance rate for the IM compared to the IV dose found in this study most likely reflects the difference in distribution rates of the IM oxytocin injected compared to

the IV. The IM plasma clearance curve did differ from the IV curve in the initial five minutes post injection, with the IM dose taking between 2-4 minutes to cause a peak in the plasma while the IV dose caused a peak that was detectable in one minute (Figures 6-1 and 6-2).

The plasma clearance rate of oxytocin in adult grey seal plasma therefore is similar to other mammalian systems studied such as the rat (0.027L/min/kg Morin *et al.* 2008) and the goat (half life of 22.3 minutes, Homeida and Cooke 1984). As no studies to date have measured the percentage of an oxytocin dose injected IM that crosses the blood brain barrier, no inferences about the potential concentrations generated centrally via this manipulation can be made. However, subcutaneous injections of oxytocin in rats have been documented to cause rises in the oxytocin concentrations present in CSF (Mens *et al.* 1983) and IM injections have been used successfully to alter behavioural frequencies in meerkats (Madden and Clutton-Brock 2011), indicating that the methodology does have the potential to succeed in penetrating the blood brain barrier. The use of IM injections rather than IV injections did not impede the delivery of the dose or the maintenance of elevated plasma oxytocin concentrations for one hour post injection, suggesting this protocol is suitable for use in the behavioural manipulation experiments outlined in Chapter 7.

6.4.2. Determination of a suitable manipulation dose for juvenile and newly weaned seals and calculating the plasma clearance rate.

The 0.00045µg/ml/kg dose of IV oxytocin was successful in significantly elevating the plasma oxytocin concentrations of newly weaned grey seals and juvenile harbour seals for one hour. This dose could therefore be used in the paired pen trial behavioural manipulations described in Chapter 7. Of the five doses tested, the 0.00045µg/ml/kg dose was the lowest to consistently elevate plasma oxytocin concentrations for a one hour period post injection. This dose additionally generated concentrations during the one hour period that were the closest to naturally elevated plasma oxytocin concentrations detected in wild free ranging individuals while associating with bond partners (Chapter 5.). As newly weaned pups or juvenile individuals can be handled and can have IV injections performed without the use of chemical immobilisation, this removed one of the practical difficulties present when manipulation adult grey seals. IV injections are preferred when possible as direct

access to the bloodstream is assured and the variation generated by the time the bolus takes to enter circulation across individuals is eliminated. IV injections, their plasma clearance rates and their effects are additionally more widely studied in the existing literature than IM injections, allowing greater foreknowledge of the potential outcomes of using such methodology to cause behavioural changes in manipulation experiments.

Plasma clearance rates for both the 0.00045μg/ml/kg dose and the lower dose of 0.00011μg/ml/kg, which consistently failed to maintain elevated plasma oxytocin concentrations in this age group of seals, were successfully calculated. Both rates were similar to each other and to the clearance rate calculated for adult female grey seals described above. Clearance studies in rats have shown a dose dependant effect on the clearance rate of plasma oxytocin, with rates increasing as the dose injected decreases (Morin *et al.* 2008) however this pattern was not detected across the three doses trialled in this chapter. This could be due to the intrinsic differences in physiology between small mammals such as rats and large ones such as grey seals, or it could be due to the different modelling methodology used in the two studies, as Morin *et al.* used one and two-compartment models to describe the various plasma clearance curves for the different doses used in contrast to this study. All detected plasma clearance rates are comparable to those found in humans (0.027 L/kg/min in men and 0.021L/min/kg in women, Leake *et al.* 1980), rats (Morin *et al.* 2008), and baboons (0.016L/min/kg, Kowalski *et al.* 1998).

The development process outlined in this chapter allowed the detection of the lowest possible dose that would still generate the prolonged elevation needed for the behavioural manipulation trials described in Chapter 7. As high oxytocin doses in manipulation experiments have been shown to attenuate behavioural changes compared to lower doses (rats; Popik *et al.* 1992, voles; Bales *et al.* 2007 and humans; Hurlemann *et al.* 2010) via desensitisation of oxytocin receptors (Phaneuf *et al.* 2000 and Robinson *et al.* 2003), detecting the lowest functional dose possible for this species was crucial. Therefore the 0.00045μg/ml/kg dose represents the best compromise between ensuring hour-long elevation and preventing un-naturally high oxytocin peaks in the plasma of the study individuals.

6.4.3. Exploration of potential of doses to affect central brain regions.

Across the three types of peripheral oxytocin dose tested in detail using clearance trials, only the lowest dose of 0.00011μg/ml/kg was significantly different to the other two higher doses (0.00045μg/ml/kg IV and 4ml 180μg/ml single bolus IM). This dose was additionally the only dose of the three to fail to keep plasma oxytocin concentrations elevated for one hour post injection, and a much greater proportion of the oxytocin peak in the plasma was cleared in the first five minutes post injection in the low dose (reduced to $1/15^{th}$ compared to the 0.00045μg/ml/kg and 4ml bolus doses which reduced the peak to a quarter and a half respectively). Low oxytocin doses given IV in rats have been reported to have faster plasma clearance rates, generate curves that fall below the detection level of the RIA within one hour and have monophasic clearance curves when compared to higher IV doses (Morin *et al.* 2008), and the clearance curves recorded for the three doses in grey seals mimic this pattern. Reduction of the plasma clearance rates of plasma oxytocin with increasing peripheral dose administered is attributed to saturation of receptors on the external surface of the endothelial cell membrane, which play an integral part in the elimination of oxytocin from circulation (Morin *et al.* 2008). Oxytocin is hydrolysed by oxytocinases after the peptide is bound to the endothelial cell membrane by its receptors (Ferrier *et al.* 1974, Tsujimoto and Hattori 2005). Therefore if the dose of oxytocin injected is large, it may contain more molecules of peptide than there are receptors to bind to them in the individual, and plasma clearance of the peptide will be slowed or halted until additional receptors become free. This would explain the persistence of elevated oxytocin concentrations in the plasma of trial individuals in the high doses but not the low dose. However it does not provide an explanation for behavioural changes observed in some studies as a result of peripheral injections.

Behavioural changes after peripheral oxytocin manipulations when compared to control trials can only occur if the brain regions responsible for those behaviours are affected by the manipulation. There are many studies stating that oxytocin cannot pass through the BBB, and as a result that no peripheral manipulation of oxytocin should be able to trigger central affects (Ermisch *et al.* 1985a, Kang and Park 2000, Churchland and Winkielman 2012). Despite this there have been many successful uses of peripheral oxytocin manipulations to alter the behaviour of rats (McCarthy

1990, Popik *et al.* 1992) voles (Bales *et al.* 2007 and Grippo *et al.* 2009), meerkats (Madden and Clutton-Brock 2011) and humans (Hollander *et al.* 2007). Explanations for such results point to small but biologically significant amounts of peripherally administered doses crossing the blood brain barrier (Madden and Clutton-Brock 2011). One study goes on to state that while the permeability of the BBB to oxytocin is poor, if a high enough dose was administered peripherally then the proportion of the dose crossing into the CSF may reach concentrations that are capable of triggering central effects, although the authors deemed this unlikely (Ermisch *et al.* 1985b). However the unique properties of the regulation system for oxytocin release in the brain may mean that even a small concentration of peptides entering the CSF can be amplified into concentrations that trigger behavioural changes. Oxytocin release is regulated via a positive feedback loop, stimulating its own release both via neuron projections to specific brain regions and into the extracellular spaces of the brain (Moos *et al.* 1984, Neumann *et al.* 1996, Landgraf and Neumann 2004). Therefore oxytocin crossing the BBB has the potential to stimulate further release of the peptide both centrally and into the peripheral bloodstream, as endogenous central oxytocin does (Neumann *et al.* 1996, Churchland and Winkielman 2012). There are only two ways to test this theory, one by labelling oxytocin molecules and directly measuring the proportion that cross the BBB after peripheral injection, and the other by using the dose and recording any behavioural changes that occur, which means central regions must have been affected by the dose.

One study to date has directly calculated the percentage of oxytocin that crosses the BBB after an IV injection in rats (Mens *et al.* 1983). This study showed that 0.002% of an oxytocin dose administered IV or subcutaneously was detectable in the CSF within ten minutes of the injection. If this figure is used in conjunction with one of the doses developed in this chapter, such as the 0.00045μg/ml/kg dose, this manipulation would result in approximately 270pg of oxytocin crossing into the CSF of seals ten minutes after being given this dose. Behavioural manipulations using central injections have been conducted with doses as low as a single 500pg injection (Dluzen *et al.* 1998), therefore this IV dose has the potential to generate CSF peaks comparable to those that have been directly injected to cause behavioural changes. The final test of this dose and the larger 4ml 180μg/ml IM injection will be its use in manipulation trials to determine if the dose successfully causes changes in behavioural frequencies when compared to control individuals given saline solution,

as described in Chapter 7. If behavioural changes occur, then the doses developed here are successful in penetrating the BBB and causing central effects in line with other existing studies utilising peripheral injections in oxytocin manipulation trials.

6.5. Conclusions

In this chapter a variety of potential doses and injection routes for peripheral oxytocin manipulations on free roaming seals was investigated. The aim was to develop peripheral doses and administration methodology that would elevate plasma oxytocin concentrations for the entire duration of behavioural manipulation trials for two different age classes of grey seal, outlined in Chapter 7. of this thesis. An IV dose for juvenile or newly weaned grey seals was successfully developed along with an IM dose suitable for use in adult female grey seals, which cannot be immobilised to permit the use of IV doses. Both doses fulfilled the requirement to significantly elevate plasma oxytocin concentrations for one hour, giving a suitable time frame for any diffusion across the BBB to take place and for any behavioural changes incurred to be recorded.

Chapter 7: Manipulation Experiments to Directly Link Oxytocin Concentrations to Behavioural Expression in Grey Seal Mothers and Newly Weaned Pups

7.1. Introduction

Chapter 5. correlated plasma oxytocin concentrations with aspects of naturally expressed maternal behaviour in grey seals. Mothers with higher oxytocin concentrations maintained lower distances between themselves and their dependant pups, and additionally performed more pup checking behaviours. While it is a vital first step to link natural behaviours to hormone concentrations, such analysis cannot be used to detect causality. To determine conclusively if oxytocin is stimulating these behaviours, experimental manipulation of the hormonal pathway must be performed to analyse the effect this has on the behaviours expressed. To date there has only been one other study on manipulating oxytocin and social behaviours in a wild species. This was performed on wild meerkats, and showed that study individuals given an IM oxytocin manipulation affected the frequency of a variety of social behaviours, including increased association with pups, increased vigilance and decreased aggression towards other adult conspecifics (Madden and Clutton-Brock 2011).
In Chapter 6. the potential problems with using peripheral manipulations of oxytocin to cause central effects were discussed, as the literature is divided on whether oxytocin can pass the BBB and trigger central effects (Viero *et al.* 2010). The current study has shown that the IV and IM doses developed for newly weaned, juvenile and adult grey seals are successful in elevating the plasma oxytocin concentrations of an individual for more than one hour (see Chapter 6.). Previous studies using peripheral oxytocin manipulations have used changes in behavioural frequencies as evidence that the IV (humans, Hollander *et al.* 2007) or IM (meerkats, Madden and Clutton-Brock 2011) manipulations were successful in penetrating the BBB and affecting central structures. This chapter will test both the practicalities and success rate of administering the developed oxytocin doses, IV or IM, to free-roaming individuals not under chemical restraint. It will also provide an opportunity to gather behavioural data to support the endocrinological evidence presented in Chapter 6. to determine the

success of the manipulations in penetrating the blood brain barrier and triggering central effects.

The aim of this chapter is to directly manipulate peripheral concentrations of oxytocin to determine which behaviours are affected by this hormone in grey seals. By using a dose of oxytocin developed in this thesis specifically for this species (Chapter 6.), the aim is to cause central effects via peripheral injections, which in turn will affect behavioural expression if oxytocin plays a role in its regulation. Both wild, free ranging mothers with dependant pups and newly weaned pups in a semi-wild pen trial setup, based on social recognition experimental designs in rodents (Bielsky and Young 2004), will be used. These two experimental designs allow testing of the effects of such manipulations in a completely wild setting and in a semi-wild environment with as many controls in effect as possible. The results of such manipulations, if successful, will allow us to link peripheral oxytocin concentrations directly to the behaviours an individual exhibits.

The objectives of this chapter were to:

1. Test the protocol developed in Chapter 6 for manipulating plasma oxytocin concentrations in newly weaned pups to levels similar to those found in dependant pups to simulate endocrine conditions they experience when with their mothers.
2. To document any changes in frequencies of approach, investigative and aggressive behaviours toward other conspecifics as a result of peripheral oxytocin manipulation in newly weaned pups in a semi-wild environment.
3. Test the protocol developed in Chapter 6 for manipulating plasma oxytocin concentrations in free ranging mothers with dependant pups.
4. To directly relate oxytocin to changes in the frequencies of investigative and interactive behaviours she exhibits towards her pup and to determine if there is a dose dependant relationship between the hormone and the behavioural frequencies mothers express on the breeding colony.

7.2. Methods

This chapter describes two separate manipulation experiments, one with newly weaned pups in a semi-wild environment and the second with free ranging mothers with dependant pups. Throughout the chapter the newly weaned pup manipulations will be described first, followed by the mother manipulations.

7.2.1. Oxytocin manipulations on newly weaned grey seal pups in a semi-wild environment.

Study site

Pen trial manipulations were performed during the 2011 breeding season on the Isle of May, from 18th November to 4th December.

Study animals

A total of 20 weaned grey seal pups were penned in two trial cohorts. The first cohort used 10 weaners penned in two groups of five for eight days. The second cohort commenced immediately after the first and also comprised of 10 weaners penned in two groups of five for eight days. All study animals were weaned within five days of their cohort and the sex ratio across the 20 individuals and the two cohorts of 10 was even. Weaned pups were deemed a suitable substitute for adults as the oxytocin receptor system in brain regions associated with social behaviours are mature at weaning in rats (Wang and Young 1997). Pups were defined as being weaned on the second consecutive day of being seen without their mother based on daily observations. Upon capture all pups were sexed, weighed and had a basal blood sample drawn. Pups were weighed ±0.2 kg on a spring balance (Salter Industrial Measurements Ltd.,West Bromwich, UK). Animals less than 30 kg were excluded from the study and early release criteria were set as a decrease in mass below 30 kg or 75% of their capture mass. This was in accordance with previous studies using captive weaned grey seal pups to prevent the post-weaning fast period being extended unnaturally (Bennet *et al.* 2007). All subjects were marked with gloss paint on the mid-dorsal region, receiving either a single letter or number depending on the pen from which they originated. Mass measurements were taken every three days

throughout the period of captivity and none of the study animals lost sufficient weight to warrant early release.

Plasma samples from free ranging weaned pups on the North Rona colony in 2009 (n=8), 2010 (n=13) and 2011 (n=15) and the Isle of May in 2010 (n=12) and 2011 (n=28) were also used to generate a measure of basal plasma oxytocin concentrations for comparative purposes.

Trial pen design

The two temporary holding pens were approximately 15 x 10 meters each and contained pools of fresh water. They were constructed with the permission of Scottish National Heritage in accordance with the Home Office licence (#60/4009) of SMRU, and were taken down once the trials were complete. Pens were located in a part of the island the breeding seals did not utilise. Individuals in one pen had never come into contact with the individuals in the other, as pups were selected based on the geographical location they were born and raised at to ensure there was no possibility of prior associations taking place. The pens were separated by 10 meters to ensure that no physical interactions could occur through the fencing outside of the recognition trials (Figure 7-1).

To conduct the paired exposure trials, a 5x5 meter trial pen was constructed out of sight from the holding pens. This was overlooked by a hide structure where observations and video recordings could be taken without disturbing the trial animals.

Figure 7-1. The two holding pens, separated by 10 meters to ensure the two groups had no contact with each other

Paired pen manipulation trial protocol

Pairs of weaned seals with no prior experience of each other were exposed to each other in a series of pen trials under the influence of an IV 0.00045µg/ml/kg oxytocin or saline injection. All study pups were allowed one rest day after capture to acclimate to the holding pens and the four other individuals with them. Paired pen trials then commenced, testing novel pairs using one animal from each pen with both animals receiving either an intravenous saline or oxytocin manipulation (Figure 7-2). The two subjects required for a trial were captured in the holding pens and administered the intravenous dose of 0.00045µg/ml/kg of either saline or oxytocin. The pair was then carried while restrained in a bag to the trial pen at the same time. Mean time spent capturing, injecting and transferring pups from the holding pen to the trial pen was 4.1 minutes (SD = 1.2 minutes). The animals were introduced into the trial pen simultaneously in a standardised manner and the trial pen was observed from a hide in real time for one hour (Figure 7-3). Video footage was also taken to allow further decoding at a later date (video camera used: Panasonic HDC-TM60 full HD 1920x1080). After the trial the animals were returned to the holding pen from which they had originated. After a rest day, the same pair was re-united in the trial pen after experiencing whichever manipulation substance (oxytocin/saline) they did not receive during their initial trial. This allowed for each pair to act as a control for itself, to help control for the substantial behavioural variation across individuals. On each trial day, five pen trials took place so that every individual from the pens was used once per day for a trial. In the 14 day period of captivity 41 paired pen trials took place, consisting of 21 oxytocin manipulations and 20 saline manipulations. This can be further broken down into 11 initial oxytocin manipulations, 10 initial saline manipulations, 10 2nd exposure oxytocin manipulations and 10 2nd exposure saline manipulations.

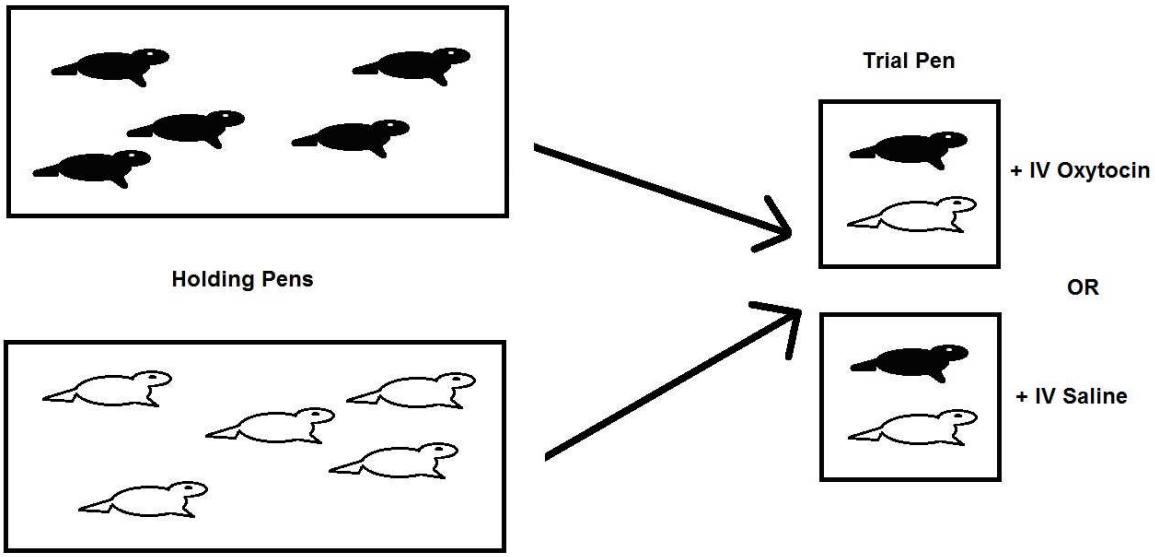


Figure 7-2. Paired pen trial design, with one individual from each pen going into the trial pen with either an IV oxytocin or saline manipulation.

Figure 7-3. Video recording of a pen trial in progress from within a hide.

Behavioural ethogram for decoding pen trial videos

The key behaviours examined during the pen manipulations were approaches, investigative and aggressive ones between the trial animals. Video footage of all trials was analysed to record the frequency of the behaviours in the following ethogram;

Approach behaviour

1. Approach – Rapid, direct locomotion towards the other trial animal, ending either within one body length of each other or involving travel over two body lengths towards the other individual.

Investigative behaviours

1. Visual check – subject raises head above ground and makes a definite movement to look specifically at the other trial subject.
2. Olfactory check – subject approaches the other study animal and extends the snout towards the other's face, anogential region or any other part of the body with nares open and vibrissae flaring. Contact with the other individual may or may not occur.

Aggressive behaviours

1. Open Mouth Threat – Head held low but above the ground, neck extended, mouth open. May be accompanied by other aggressive behaviours (see below).
2. Vocalisation – Any hissing, growling or howls while interacting with the other trial animal.
3. Flippering – One or both fore flippers waved in a rapid manner with claws usually extended at the other animal, may or may not come into contact.
4. Lunge – typically an attempt to bite the other individual, rapid, aggressive neck extension and retraction with snapping or open mouth threat with no contact with the other individual.
5. Bite – Actual physical contact while biting to any part of the other animal's body, in a rapid and aggressive manner.
6. Startle – violent twitching motion, a physically visible startle response to one pup approaching, touching or vocalising at the other trial animal.
7. Flee – Rapid locomotion away from the other trial animal, resulting in separation of more than one body length between the two individuals.

Actions fitting these categories were tallied for all trial animals, giving a score for the number of checks (investigative behaviours), approaches and aggressive behaviours per individual, per trial. The amount of time the trial animals spent within one body length (using the weaned pup metric) of each other was recorded. Colonially breeding adult grey seals typically maintain distances of 2 adult body lengths between themselves and neighbours (Pomeroy *et al*. 1994, Twiss *et al*. 2000) therefore it is

assumed any individual within one body length of another would be sufficiently close to evoke a response from the other animal.

Plasma sampling and analyses

All samples were obtained using the capture, restraint, sampling and laboratory protocol detailed in Chapter 2. Plasma samples were taken when the pups were captured from the wild, just before entry into the pen environment. This was done to ensure an abnormal group of study animals had not been selected for these trials and to deduce the success of the oxytocin manipulations in raising the plasma concentration significantly above basal. After a subset of both types of manipulation trials (trials sampled: n=4 post oxytocin and n=4 post saline, giving individuals sampled: n=16) plasma samples were taken immediately at the end of the 1 hour trial and before the pups were returned to the holding pens to investigate the efficiency of the manipulation. Post-saline samples were taken to ensure that no rise in oxytocin occurred from the injection procedure, which has been hypothesised to occur by other researchers (Devarajan and Rusak 2004). Repeated sampling through out the captive period to detect any effect of captivity on plasma oxytocin was not done in these individuals. This was based on the findings of the previous year's pen trials (2010) which found no evidence of such an effect during these experiments (Chapter 8.). All samples were then stored, processed and analysed as outlined in Chapter 2. Inter-assay coefficient of variance over the six plates used in this study was 5.7%.

Statistical analyses

For all ANOVAs, Tukey honest significant differences post-hoc test were performed to determine which treatments were different from each other when appropriate. Data on the basal concentrations of plasma oxytocin in grey seal weaned pups from the Isle of May and North Rona colonies were compared by a one-way ANOVA to the study group's oxytocin concentrations at capture to check that a physiologically unusual group had not been accidentally selected for these trials. The data were analysed after a square root transformation as the original data were not normally distributed when subjected to a Shapiro Wilk test ($p=0.007$).

Plasma oxytocin concentrations from post-trial samples for both saline (postSAL) and oxytocin (postOT) treatments were compared by a one-way ANOVA to the basal concentrations for the study group when not in a trial (basal) to determine the success

of the oxytocin manipulations and if the saline manipulations had any non-specific effects on plasma oxytocin concentrations. The data were analysed after a natural log transformation as the original data was not normally distributed when subjected to a Shapiro Wilk test ($p<0.001$).

Generalised additive mixed models (GAMs) (Wood 2006a) were used to analyse the behavioural tally data for the frequency of approach, investigative and aggressive behaviours and time spent in within one weaned pup body length of each other. Variables included in these models were the type of manipulation and the sequence they were given to a pair (oxytocin first: OT1, saline first: Sal1, oxytocin second: OT2, saline second: Sal2), sex of the focal animal, the behavioural frequencies recorded that were not the dependant variable in the current model (approaches, investigations, aggression and time spent within one body length) and time spent in captivity (see Appendix B, Table B-1). Models were fitted using the multiple generalized cross validation library mgcv (Wood, 2012). The identities of both individuals in the trials were fitted as two random effects smooths (focal and response animal) (Wood 2006b) to control for pseudo-replication in the dataset from using the same individuals over multiple trials. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). The models for approach and aggressive behaviours were fitted with Poisson error distributions with log links using the multiple generalized cross validation library mgcv (Wood 2006a) due to the presence of zero values in the data, while the models concerning investigative behaviour and time spent in one body length were fitted with a Gaussian distribution. Model selection was done by backwards stepwise elimination through examination of R^2 values, QQ and residual plots to identify the best model for the data.

7.2.2. Oxytocin manipulations on free ranging mother grey seals with dependant pups.

Study site

Free ranging manipulations on single mother-pup pairs (total n=13) were performed on the North Rona colony from the 7th October to 21st October 2011 (n=6) and on the Isle of May from the 23rd November – 3rd December 2011 (n=7).

Study animals

Free-range manipulations required selection of mothers using the following criteria to maximise the success of the experiment and ensure that data could be collected from the trials.

i) All mothers had a dependant pup at the time of the experiment.

ii) All mothers had to be in a location that permitted an observer to record data from the trials while being hidden from the study females, as it was not possible to build hides for locations on the Isle of May colony due to the wide range of locations across the island that suitable mother-pup pairs could be found. Mothers on the North Rona colony were all observed and recorded from the hide site (see Chapter 2.).

iii) All mothers had to be on the periphery of the main colony so that the manipulations would not cause widespread disturbance to individuals not involved in the trial during the repeat approaches to administer doses. It also meant that it was less likely that manipulated mothers would be distracted by other conspecifics during the 1 hour trial.

iv) All mothers had pups of similar age (stage 2, Kovacs 1987) at time of first manipulation.

Free ranging manipulation protocol

Mother-pup pairs identified as suitable for these trials were observed for 30 minutes prior to manipulation to ensure that no disruptive events (such as an attempted copulation by a male) occurred immediately prior to the trials. At the start of the trial, mothers were given a 4ml 180µg/ml dose of oxytocin or saline intramuscularly (see Chapter 6.). This was performed by one researcher approaching close enough to allow a syringe to be discharged into the flank region while mounted on a jab stick. The researcher then retreated and the mother-pup pair were recorded using video camera equipment (video camera used: Panasonic HDC-TM60 full HD 1920x1080) for one hour from distances ranging from five meters away to 200 metres. Video recordings included the dose administration procedure to allow accurate timing of trials when decoding them. After the initial manipulation on the first day of the experiment, the pair had a rest period of one day. On the third day of the experiment mothers were administered whichever treatment (oxytocin/saline) had not been given during the first exposure to ensure each pair would have its own control for comparison. In one

trial day, two to three mother-pup pairs were manipulated and the manipulation they received first (oxytocin or saline) was randomised to control for order effects.

Behavioural observations and decoding

The key behaviours examined during the free range manipulations were the frequencies of the mother investigating her pup ('pup checking') and interacting with her pup. Video footage of all trials was decoded for one hour starting from 15 seconds after the intramuscular injection. While decoding the following ethogram was used to document the key investigative and interaction behaviours alongside aggressive behaviours and her alertness to her surroundings;

Investigative behaviours

1. Visual check – Mother raises head above ground and makes a definite movement to look specifically at her own pup.
2. Olfactory check – Mother approaches the her pup and extends the snout towards the pup's face, anogential region or any other part of the body with nares open and vibrissae flaring. Contact with the other individual may or may not occur.

Interactive behaviours

1. Flippering – stroking of the pup's head or any other part of it's body with a fore flipper to encourage suckling. Typically slow but can vary, however never aggressive to the point the pup flees from the mother.
2. Interact with pup – allowing pup to mouth, bite and flipper mother's head, fore-flippers or any other part of her body.
3. Crush – moving to lie on top of the pup.

Aggressive behaviours

1. Open Mouth Threat – Head held low but above the ground, neck extended, mouth open. May be accompanied by other aggressive behaviours (see below).
2. Vocalisation – Any hissing, growling or howls while interacting with the other trial animal.
3. Flippering – One or both fore flippers waved in a rapid manner with claws usually extended at the other animal, may or may not come into contact.

4. Lunge – typically an attempt to bite the other individual, rapid, aggressive neck extension and retraction with snapping or open mouth threat with no contact with the other individual.
5. Bite – Actual physical contact while biting to any part of the other animal's body, in a rapid and aggressive manner.

Alert behaviours

1. Visual check – Mother raises head above ground and makes a definite movement to look specifically at an aspect of her surroundings, including other conspecifics but not including her own dependant pup (see 'investigative behaviours' above).

Actions fitting these categories were tallied for all trial animals, giving a score for the number of 'checks' (investigative behaviours), interactions with pup, aggression and alerts per mother, per trial. The amount of time mothers spent in the following conditions was also recorded;

1. Time spent within 1 adult body length of pup (mother-pup distance).
2. Time spent either in physical contact with the pup or with the pup less than half an adult body length from the mother's head.
3. Time spent presenting nipples to the pup to suckle, and nursing.
4. Time spent resting, with the head down and no movement, with eyes open or closed.

Plasma sampling and analyses

All samples were obtained using the capture, restraint, sampling and laboratory protocol detailed in Chapter 2. Plasma samples were taken from a subset of the mother-pup pairs in the free-ranging manipulation trials post oxytocin manipulation to test the efficiency of the jab stick protocol and the success of the 4ml 180µg/ml dose in elevating oxytocin concentrations for the one hour trial (n=5). Plasma samples were additionally taken from a subset of mother-pup pairs to ensure concentrations returned to basal four hours after the manipulation took place (n=5).

Statistical analyses

Basal oxytocin concentrations were calculated for free roaming mothers during the first seven days of lactation from the North Rona colony in 2011 (n=6). These were compared by a one-way ANOVA to plasma oxytocin concentrations from a sub-set of post-trial samples from the oxytocin (postOT) manipulations and samples taken at the three hour mark post trial (ThreeHourPostOT) (n=5 for both). This would confirm the success of the oxytocin manipulations in the one hour trial and confirm that the manipulation did not persist for a large amount of time post trial. The data were analysed after a reciprocal transformation as the original data were not normally distributed when subjected to a Shapiro Wilk test ($p<0.001$). Tukey honest significant differences post-hoc test were performed to determine which treatments were different from each other when appropriate.

Generalised additive mixed models (GAMs) (Wood 2006a) were used to analyse the behavioural tally data for the frequency of investigative and interactive behaviours and time spent in within one body length of each other, time spent in contact with the pup and time spent nursing the pup. Biologically plausible variables included in the selection process for these models were; the type of manipulation and the sequence they were given (oxytocin first: OT1, saline first: Sal1, oxytocin second: OT2, saline second: Sal2), the behavioural frequencies recorded that were not the dependant variable in the current model and the colony the trial was conducted on (see Appendix B, Table B-2). Models were fitted using the multiple generalized cross validation library mgcv (Wood, 2012). The identities of mothers in the trials were fitted as a random effects smooth (Wood 2006b) to control for pseudo-replication in the dataset from using the same individuals over multiple trials. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). The model on interaction behaviours was fitted with a Poisson error distribution with log links due to the presence of zero values in the data, while the models for investigative behaviour, time spent in one body length of the pup, time spent in physical contact with the pup and time spent nursing the pup were fitted with a Gaussian distribution. Model selection was done by backwards stepwise elimination through examination of R^2 values, QQ and residual plots to identify the best model for the data.

7.3. Results

7.3.1. Oxytocin manipulations on newly weaned grey seal pups in a semi-wild environment

Behavioural frequencies and trial type

Across the four GAMs, the manipulation where saline was given first (Sal1) to a pair resulted in trials that had fewer approaches ($p=0.02$), more investigations ($p=0.003$), more aggression ($p<0.001$) and less time spent within one body length ($p=0.002$) between the two individuals in the trial compared to pairs given oxytocin in their first trial (OT1) (Tables 7-1, 7-2, 7-3 and Appendix B, Tables B-3 and B-4). When given the second treatment, both the saline and oxytocin manipulations had fewer approaches compared to either of the first manipulations ($p<0.001$ and $p=0.01$ respectively) but had similar amounts of investigative behaviour and spent the same amount of time within one body length as trials where oxytocin was given first. With aggressive behaviour, the lowest frequencies of aggression were in trials where pairs received the oxytocin manipulation second ($p=0.005$) having already encountered the other study animal in the first saline trial. Figures 7-4 to 7-7 show the various manipulations and the behavioural changes they caused.

Table 7-1. Mean values with standard errors for the frequencies of approaches, investigative behaviour, aggressive behaviour and the time spent within one body length of the response pup for each type of manipulation.

Manipulation type	Approaches	Investigative behaviours	Aggressive behaviours	Time in one body length (hours:minutes:seconds)
OT1	8.5 (±0.3)	51.5 (±4.9)	17 (±0.5)	00:44:12 (± 00:04:12)
Sal1	7.2 (±0.2)	67.7 (±4.6)	27.3 (±0.2)	00:32:35 (± 00:03:58)
OT2	5.8 (±0.1)	46.9 (±4.7)	15.8 (±0.2)	00:44:38 (± 00:04:02)
Sal2	5.3 (±0.1)	48 (±4.5)	19.8 (±0.09)	00:44:25 (± 00:03:58)

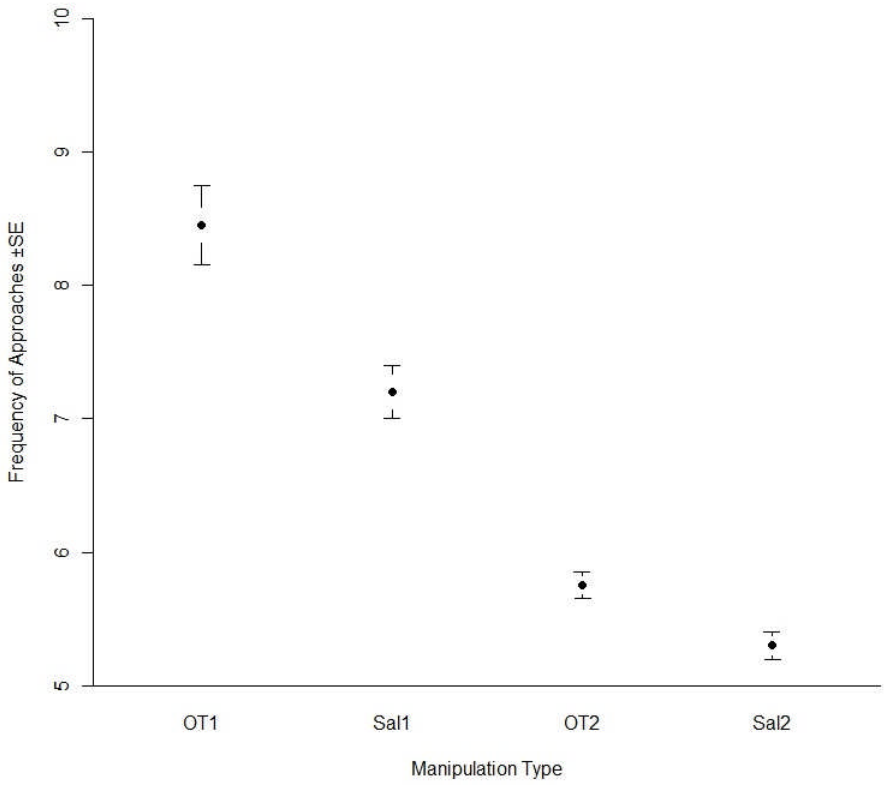


Figure 7-4. Mean frequency of approaches across the four types of manipulation trial with error bars.

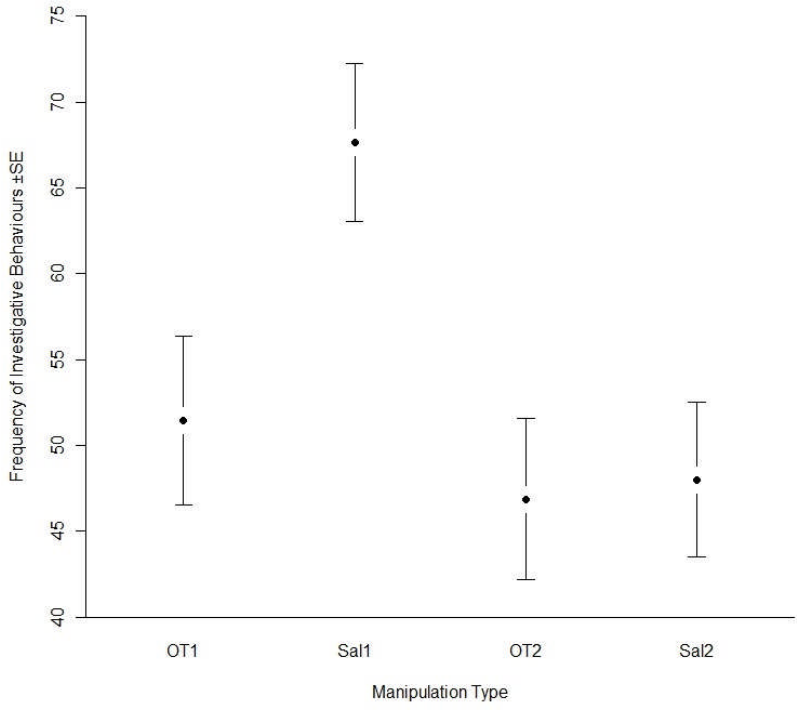


Figure 7-5. Mean frequency of investigative behaviours across the four types of manipulation trial with error bars.

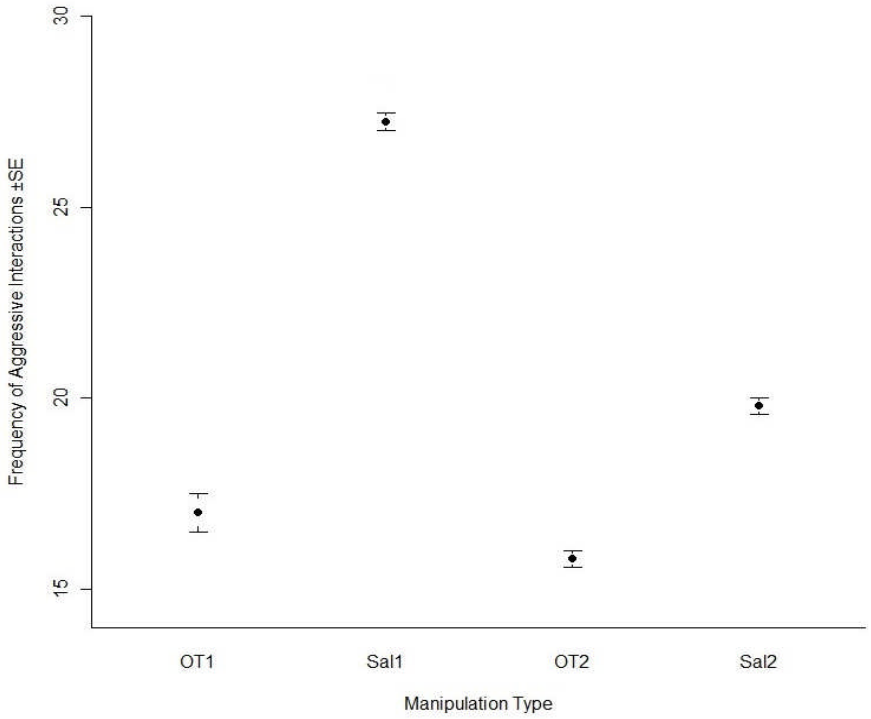


Figure 7-6. Mean frequency of aggressive behaviour across the four types of manipulation trial with error bars.

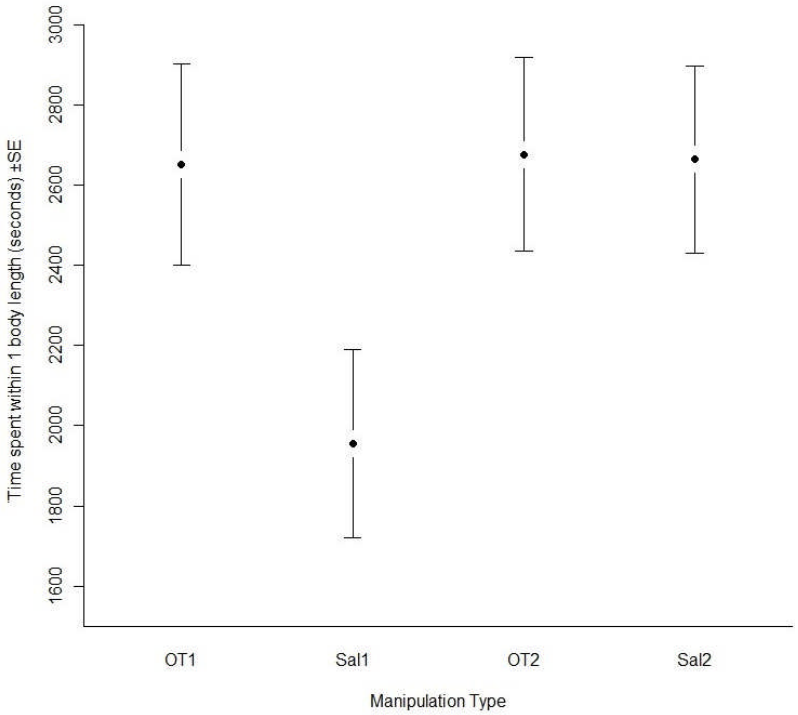


Figure 7-7. Mean time spent within one body length of the response animal across the four types of manipulation trial with error bars.

Table 7-2. Significant fixed effect variables from the four GAMs for frequency of approach, investigative, aggressive behaviour and the time spent within one body length, their estimates, standard errors and p values.

Dependant variable	**Explanatory variable**	**Estimate**	**Standard Error**	**P value**
Approaches	Trial type (Sal1)	-0.4	1.6	0.02
	Trial type (Sal2)	-0.5	0.1	<0.001
	Trial type (OT2)	-0.4	1.5	0.01
	Investigative behaviour	0.03	0.003	<0.001
	Aggressive interactions	-0.01	0.003	<0.001
Investigative	Trial type (Sal1)	13.9	4.6	0.003
	Approaches	1.9	0.3	<0.001
	Aggressive interactions	0.5	0.08	<0.001
Aggressive interactions	Trial type (Sal1)	-0.6	0.2	<0.001
	Trial type (OT2)	-0.4	0.2	0.005
	Time in captivity	-0.1	0.02	<0.001
	Time spent within 1 body length	0.0009	0.00005	<0.001
	Approaches	-0.03	0.008	<0.001
	Investigative behaviour	0.02	0.002	<0.001
Time spent within 1 body length	Trial type (Sal1)	-753.9	233.8	0.001
	Time in captivity	84.7	37.9	0.03

Table 7-3. Random effect variables from the two GAMs where they had a significant effect on the model and their standard deviations and p values.

Dependant variable	**GAMM random effect**	**Standard deviation**	**P value**
Approaches	Focal Individual	0.4	0.02
	Response Individual	0.3	0.3
Aggressive interactions	Focal Individual	1.2	<0.001
	Response Individual	1.3	<0.001

Effect of intravenous oxytocin manipulation

There were significant differences across the three ('basal', 'postOT' and 'postSAL') treatment groups ($F_{(2, 33)} = 26.4$, $p<0.001$). Plasma oxytocin concentrations were significantly elevated in the 'postOT' treatment (mean= 27.9 ±5.6 pg/ml) when compared to basal (mean = 7.3 ±1.5 pg/ml, $p<0.001$) and postSAL (mean = 6.6 ±1.3 pg/ml, $p<0.001$) concentrations. There was no difference between concentrations in individuals in the postSAL and basal groups ($p=0.8$) (Figure 7-8).

Basal oxytocin detection in the study group

There were significant differences between samples taken from different colonies/ study cohorts (North Rona: NR, Isle of May: IoM, pen trial study group: Pen) and years (2009, 2010 and 2011) ($F_{(6, 106)} = 10.9$, $p<0.001$) (Table 7-4 and 7-5). There were no significant differences between the study group individuals (mean = 7.5 ±0.75 pg/ml) and the free roaming individuals from the Isle of May in 2011 (mean = 10 ± 0.85 pg/ml, $p=0.06$). However there were significant differences between some free-ranging groups in some sampling years and across the two colonies, including significant differences between the study group for pen trials conducted in 2010 (mean=14.4 ±2.2 pg/ml, Chapter 8.) and the study group in 2011 ($p<0.001$) (Table 7-5 and Figure 7-9).

Table 7-4. Mean values with standard errors of the basal plasma oxytocin concentrations detected in seven study groups of weaned pups collected across three cohorts (two from free ranging colonies, one from the pen trials cohorts) and three years.

Year	North Rona (NR)	Isle of May (IoM)	Pen Trials Study Group (Pen)
2009	8.12 (±0.8)	NA	NA
2010	9.31 (±0.85)	13.76 (±1.05)	14.38 (±2.2)
2011	12.58 (±1)	10.02 (±0.85)	7.52 (±0.75)

Table 7-5. Significantly different study cohorts of weaned grey seals.

Cohort 1	Cohort 2	P value
NR09	IoM10	0.004
NR09	Pen10	<0.001
NR09	NR11	0.03
NR10	IoM10	0.02
NR10	Pen10	0.002
NR11	NR09	0.03
NR11	Pen11	<0.001
IoM10	IoM11	0.03
IoM10	Pen11	<0.001
IoM11	Pen10	0.001
Pen10	Pen11	<0.001

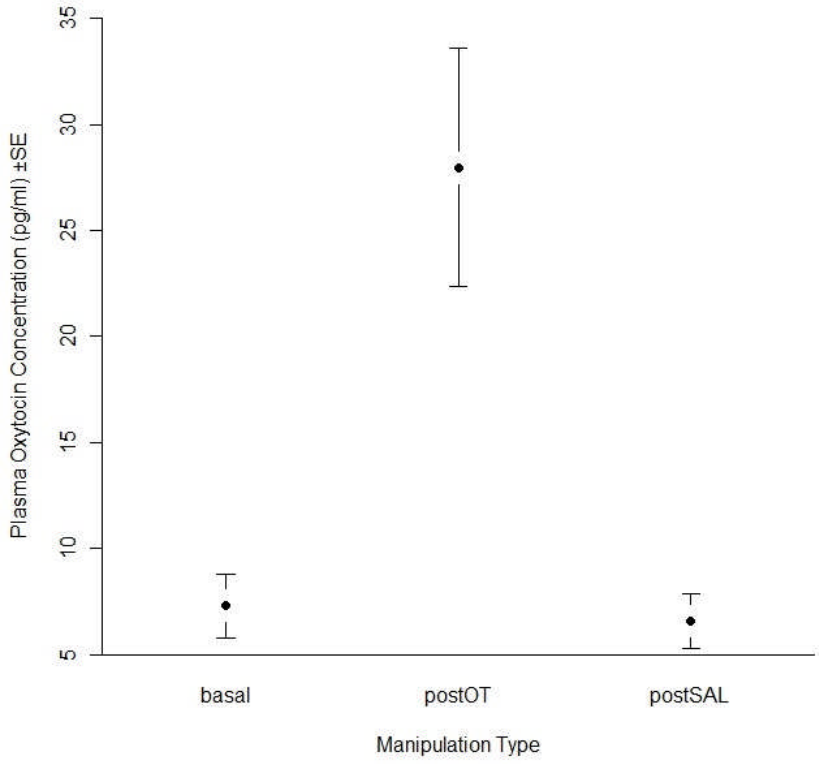


Figure 7-8. Mean plasma oxytocin concentration (pg/ml) across the two types of manipulation (postOT and postSAL) and weaned pups not in a trial environment (basal) with error bars.

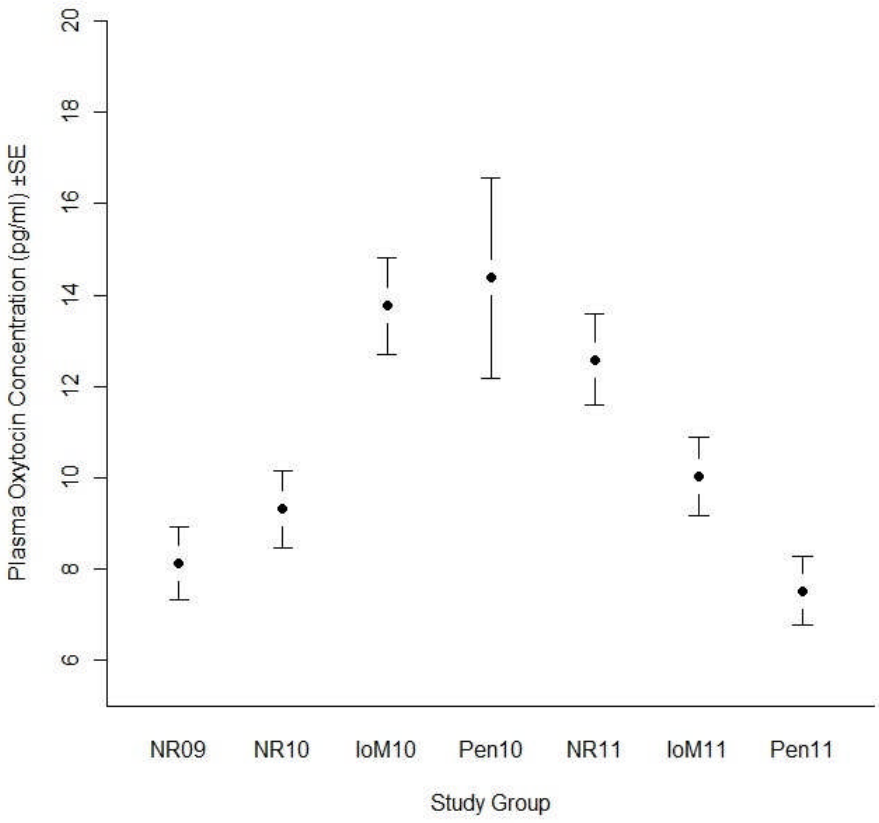


Figure 7-9. Mean plasma oxytocin concentration (pg/ml) in the 2011 study group (pen11) and free roaming weaned pups on the Isle of May in 2011 (IoM11) with error bars.

7.3.2. Oxytocin manipulations on free ranging mother grey seals.

Behavioural frequencies and trial type

Across the five GAMs, type of manipulation or the order in which they were given to an experimental pair did not significantly affect the amount of investigative (p=0.9) or interactive (p=0.5) behaviours. It additionally did not significantly effect the time a mother spent within one body length of her pup (p=0.7), in physical contact with her pup (p=0.5) or nursing her pup (p=0.7). Therefore there was no clear response to the manipulations in the mother grey seals (Appendix B, Tables B-5 and B-6).

Effect of intramuscular oxytocin manipulation

There were significant differences across the three ('basal', 'postOT' and 'ThreeHourPostOT') treatment groups ($F_{(2, 16)} = 8.5$, p=0.003). Plasma oxytocin concentrations were significantly elevated in the 'postOT' treatment (mean= 128.5 ±33.3pg/ml) when compared to basal (mean = 6.9 ±1.5 pg/ml, p=0.002) and ThreeHourPostOT (mean = 9.9 ± 3.2pg/ml, p=0.05) concentrations. There was no difference between concentrations in individuals in the ThreeHourPostOT and basal groups (p=0.4) (Figure 7-10).

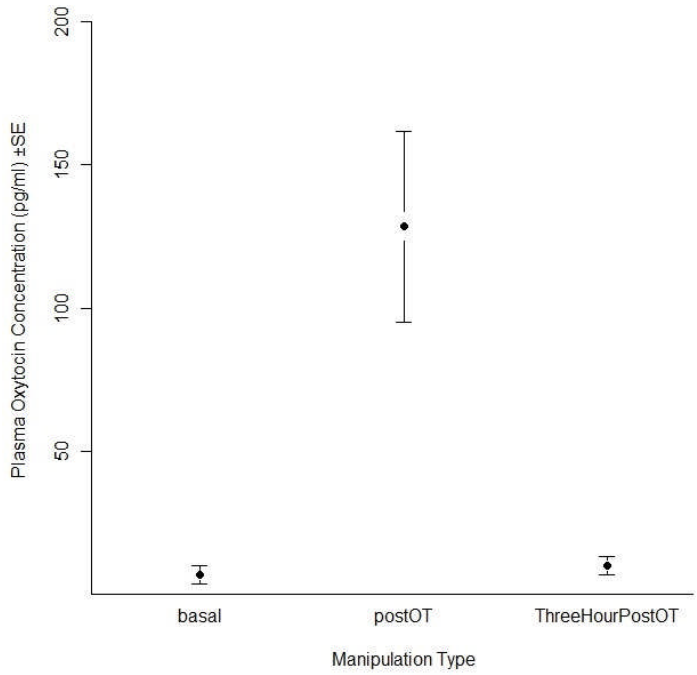


Figure 7-10. Mean plasma oxytocin concentration (pg/ml) across the two post trial sampling events (postOT and ThreeHourPostOT) and in animals not in a trial environment (basal) with error bars.

7.4. Discussion

7.4.1. Oxytocin manipulations on newly weaned grey seal pups in a semi-wild environment

Behavioural frequencies and trial type

Oxytocin manipulations on weaned grey seal pups with no prior experience of each other impacted on the behaviours that were expressed during the initial meeting when compared to individuals given a saline treatment. Pups given oxytocin prior to trials showed greater amounts of pro-social behaviour, approaching the other study individual more and spending more time within one body length of each other than those treated with saline. Additionally, despite increasing the time in close proximity to each other, oxytocin treated individuals showed less aggressive behaviour than saline treated individuals, and did not investigate the other individual as much despite having never encountered the other study pup before. These results are in agreement with the correlations found between natural plasma oxytocin concentrations and behaviour in free ranging grey seal mothers as discussed in Chapter 5. Time spent in close contact with another individual in a trial setting has been cited as a sensitive indicator of social preference in rodent manipulation trials (Cho *et al.* 1999). These findings, therefore, show that high plasma oxytocin causes individuals to maintain close proximities to each other, a behaviour that has great importance in preventing mother-pup separation on a breeding colony.

The decreases in investigative and aggressive behaviours detected in this chapter have parallels in the findings from free ranging, naturally expressed behaviour and oxytocin concentrations, as discussed in Chapter 5. Dependant pups decrease the amount of time they spend alert to their surroundings when with mothers with high plasma oxytocin concentrations. The only dependant pup observed to persistently exhibit aggression towards its mother was subsequently abandoned, and the only mother observed to exhibit aggression consistently towards her own pup adopted a non-filial pup to nurse alongside her own (KJ Robinson, pers. obs). Therefore oxytocin plays an important role in stimulating pairs of individual grey seals that share a positive bond to remain together, such as the mothers and dependant pups. It additionally prevents aggression from being directed towards the bond partner, which

would have a negative impact on the bond and may result in costly behaviours such as abandonment or adoption.

There are many examples of oxytocin manipulations in mammalian species increasing the amount of pro-social behaviours exhibited between individuals. Many studies use injections into specific central brain regions to guarantee increased oxytocin concentrations in the structures the study is investigating, and such injections have stimulated the formation of social bonds and preferences in voles (Insel 1992, Cho *et al.* 1999) and facilitated social recognition via reduced investigation frequencies in rats (Popik *et al.*1992). Intranasal administration of oxytocin is also widely considered to rapidly reach central brain regions (Churchland and Winkielman 2012), and increases the time spent in contact with a novel partner and partner driven social behaviour in captive Black-pencilled marmosets (*Callithrix penicillata,* Smith *et al.* 2010). However in this study IV injection was used as part of the trial methodology, potentially complicating comparisons to this work due to the debate in the literature over whether peripheral oxytocin manipulations are suitable for affecting central brain structures and the behaviours they promote (Churchland and Winkielman 2012). The endocrinological evidence supporting BBB transmission from peripheral to central regions is discussed in Chapter 5, the behavioural results presented in this chapter provide support for these arguments. In this experiment, the IV injections did result in behavioural changes in the study individuals compared to those receiving saline manipulations. Therefore, central regions must have been affected by the peripheral manipulation, either via permeability of the BBB to plasma oxytocin or stimulation of peripheral receptors that then triggered central neurotransmitter release that caused the behavioural changes (Madden and Clutton-Brock 2011). Other studies using peripheral oxytocin manipulations have documented similar results, with IV injections in humans (Hollander *et al.* 2007), subcutaneous injections in voles (Grippo *et al.* 2009) and IM injections in wild meerkats (Madden and Clutton-Brock 2011) all resulting in significant changes in expressed behaviour compared to controls. Single interperitonial injections of oxytocin have also been shown to significantly impact on the development of receptor distributions in central brain regions in neonatal voles (Bales *et al*. 2007). Therefore, while the mechanisms behind how such peripheral oxytocin manipulations effect central brain regions remain unclear, there is compelling behavioural evidence in the literature to suggest this does occur, which the findings from manipulations on weaned grey seal pups agree with.

Interestingly in this study an order effect was observed in the weaned pup manipulations. While there was strong differences dependant on manipulation type in the first trial a pair underwent, there was no difference in the number of approaches, number of investigations or the time spent within one body length of each other in the second trial regardless of manipulation type given. There are two potential explanations for this result. The pups could naturally be capable of forming a stable social memory of the other conspecific they encountered in the trial after no more than one hour of close proximity to them. Alternatively the oxytocin manipulation given in the first trial artificially accelerated the formation of a social memory in the two individuals, so when they met again in the second saline exposure trial a positive social bond was already in place. If either of these explanations is true, then the individuals would not have considered each other as novel individuals and would not have displayed behavioural frequencies similar to those seen in the initial trial with saline manipulations. Unsurprisingly the trial pairs given saline first and oxytocin second offer no insight into this problem; during the second trials the individuals had experienced both a one hour exposure to each other prior to the experiment and were under the influence of a manipulation know to change behavioural expression when encountering strangers. Therefore with the current dataset it is impossible to distinguish between the two possible explanations for the lack of behavioural differences across the second trials.

The use of the same pairs twice, for a saline and for an oxytocin manipulation, in these experiments attempted to control for individual variations in behaviour which may have biased the trials, especially as grey seals have been shown to exhibit consistent individual differences in behaviour (Twiss *et al.* 2012a and 2012b, Culloch 2012). The initial trial was only one hour in duration, and the individuals were subsequently separated and had no contact until the second trial two days later. It was therefore assumed that individuals in the second trial would act as if the pair was still novel to each other, affected only by the manipulation given to them. Mother grey seals on the Isle of May lack the ability to recognise their own filial pups acoustically (McCulloch *et al.* 1999), therefore, there was no reason to think that weaned pups only exposed to each other for one hour would form stable, long term memories of each other. Oxytocin manipulations that affect central brain regions have been documented to accelerate the formation of social memory in other species though, making this a more plausible explanation for the homogeneity of the results in the

second manipulation a pair underwent regardless of the type of solution (oxytocin/saline) used. Injections into central brain regions in mice enable social memory acquisition (Ferguson *et al.* 2001) stimulates the formation of social bonds and preferences in voles (Insel 1992, Cho *et al.* 1999), increases retention of social memory in rats (Dluzen *et al.* 1998) and in humans has been shown to accelerate the process of social memory formation (Savaskan *et al.* 2008, Guastella *et al.* 2008) and to increase the retention of social memories (Rimmele *et al.* 2009). IV injections of oxytocin have additionally been found to produce behavioural changes that persisted across at least 16 days between trials in humans (Hollander *et al.* 2007). Therefore it seems probable that oxytocin manipulations are driving the persistence of pro-social behaviour expression in the second trials. This can only be conclusively proved if trials documenting the natural speed of social memory formation are conducted on weaned grey seal pups, which was not within the scope of this thesis. However a series of pen trial experiments testing this concept are due to be conducted in the 2013 breeding season on the Isle of May to address these phenomena in our results.
The weaned pup pen trials allow us to conclusively link high plasma oxytocin concentrations to aspects of pro-social behaviour, which have been shown to be correlated to each other in wild, free-ranging mothers with dependant pups (Chapter 5.). Plasma oxytocin stimulates individuals to remain within close proximity of each other, while decreasing the aggression displayed towards the bond partner. Oxytocin manipulations in weaned pups additionally accelerate the acquisition of social memory and familiarity with another individual, lowering the investigative behaviour and aggression expressed towards a novel individual, and this memory of the other individual appears to be stable across two days once it has formed.

Effectiveness of intravenous oxytocin manipulation

While in the one hour pen trials, individuals given the IV oxytocin injection had significantly elevated plasma concentrations for the entire time in the trials while those given a saline injection had no change from basal concentrations. Additionally, after one hour the manipulation raised the plasma oxytocin to a mean 27.9 ±5.6 pg/ml, comparable to those experienced naturally in pups during the dependant period (mean = 21.9 ±2.2pg/ml, Chapter 5.) and in mothers performing behaviours typically associated with oxytocin release such as nursing (35.2pg/ml, Chapter 5.).

The ability of peripheral oxytocin injections to have central effects, and so cause changes in the behaviour that individual exhibits, has long been debated in the literature and is discussed in detail in Chapter 6. These trials provide the behavioural evidence to support the endocrinological evidence discussed in Chapter 6. that such manipulations do penetrate the BBB despite being commonly cited as impermeable to oxytocin transfer (Ermisch *et al.* 1985a, Kang and Park 2000). The dose generated and the manipulation methodology used for the weaned pup manipulations was therefore a success, resulting in sustained endocrinological and behavioural changes across the one hour trials.

Basal Oxytocin Detection in the Study Group

There was no difference between the basal oxytocin levels of the captive group and free-roaming weaned pups on the Isle of May in 2011, indicating that we had not sub-sampled an unusual and unrepresentative group from the study. However there were differences between some basal datasets from different years of sampling and different colonies. Plasma oxytocin concentrations were consistent across the three years of the study on North Rona, however on the Isle of May there were significant differences between the 2010 and 2011 seasons. Additionally the 2010 concentrations from the Isle of May were significantly higher than those found on North Rona, while in 2011 there was no difference between the two colonies. As oxytocin concentration in weaned individuals is most likely dependant on time since the weaning event and last contact with the mother (see Chapter 5.) this is most likely due to accidental sampling of a younger subset of weaners in the 2010 year on the Isle of May compared to the other subsets gathered in 2011 and on North Rona. This is reflected in the difference between the study groups in the pen trials during 2010 (Chapter 8.) and 2011. Individuals in the 2010 trails were penned within a shorter time period since weaning (mean=2 days post wean, SD = 1.1 days) compared to the 2011 group (mean=3 days post wean, SD = 1.5 days), which were significantly different to each other with the 2010 group (mean = 14.4 ±1.5 pg/ml) having higher concentrations than the 2011 group (mean = 7.5 ±0.8 pg/ml). The GLM analysis in Chapter 5. shows that when modelled with life history stage, sex, colony and sampling year there is no significant difference in plasma oxytocin in weaned pups between colonies or sampling years, supporting the theory that these small datasets do not represent real differences between the two colonies.

7.4.2. Oxytocin manipulations on free ranging mother grey seals with dependant pups.

Behavioural frequencies and trial type

Oxytocin manipulations on grey seal mothers with dependant pups had no impact on the behaviour they expressed during the one hour trial period. This is in direct contrast to the successful weaned pup manipulations described above, where behavioural changes consistent with naturally observed behaviour and oxytocin correlations occurred. There were several differences between the methodology for the pen trial manipulations and the free-ranging manipulations, which seem the most likely cause of the complete lack of behavioural changes in these experiments. The route of injection and dosage given in these trials both had to be altered from the doses developed for weaned pups in Chapter 6. due to constraints of working with large, aggressive, adult free-ranging animals. Endocrinological evidence (discussed below and in Chapter 6.) suggests that central brain structures were affected by the oxytocin dose injected for mothers, despite using an IM route of administration. IM manipulations have also been used successfully to manipulate free-ranging mammalian behaviour in a previous study on meerkats (Madden and Clutton-Brock 2011). Therefore, the route of administration (IM rather than IV as in the weaned pup trials) is unlikely to be the source of the problem, leaving the actual dose given to mothers as the most likely cause of the failure of these manipulations.

Developing a peripheral dose for free-ranging mothers was much harder than for weaned pups. There was no opportunity to gain access to mother grey seals outside of the breeding season, and on breeding colonies it was impossible to process and analyse samples. This prevented assessment of the results of the manipulations while the opportunity to adjust and trial higher or lower doses was available. The uncertainty of whether peripheral manipulations would work at all at the time of the original serial sampling trials led us to use a dose that was suitable to provoke a physiological response from the females, i.e. caused milk letdown. This dose of 1ml IV consistently triggered milk letdown in all females that received it regardless of maternal mass at injection. The mass of an individual is typically crucial information when developing a suitable dose. However, maternal mass in grey seals varies hugely across the dependant period due to mobilisation of blubber reserves to produce lipid

rich milk (Haller *et al.* 1996) and is an unreliable measure of the critical physiological mass required to accurately determine doses. Additionally the methodology for administering the dose in free-ranging manipulation studies did not entail drugging the female to perform the manipulation, and mothers cannot be weighed without being chemically immobilised. This prevented a measure of the mother's mass from being gained before the trial commenced, which contrasts greatly to the weaner manipulations, in which individual body masses and rates of mass loss were measured and each manipulation injection tailored to the individual that was receiving it. These factors lead to the selection of a dose of 1ml 180μg/ml IV, which when converted using mean mother mass at parturition (210.6kg, Haller *et al.* 1996) gives on average an IV dose double the 0.00045μg/ml/kg dose developed for the IV weaned pup manipulations. Finally, the only advice on converting IV doses to IM doses is to multiply the IV dose by four (Intervet UK Ltd). This gave the standard 4ml IM injection that was used for all mother manipulations.

The detailed serial sampling data collected from the dose development experiments (Chapter 6.) shows that over the hour of the trials, plasma oxytocin concentrations in manipulated mothers were approximately double that of manipulated weaned pups. Exposure to high dose oxytocin manipulations have been documented to attenuate behavioural responses to the hormone, as seen with doubling doses of centrally administered oxytocin in rats (Popik *et al.*1992), increasing doses of intranasal manipulations in humans (Hurlemann *et al.* 2010) and doubling doses of interperitonial injections in voles (Bales *et al.* 2007). Why this occurs is attributed to oxytocin receptors desensitising to the hormone's presence during prolonged elevation (Phaneuf *et al.* 2000). In vitro myometrial cells exposed to oxytocin desensitise to the hormone, with inactivation of half the cell population occurring at 4.2 hours (Robinson *et al.* 2003). While the doses given to grey seal mothers were sufficiently low as to clear from circulation within three hours of the IM injection, and the trial itself lasted only the first hour from the injection, it is possible that high oxytocin exposure may have desensitised a sufficient proportion of receptors in the mothers to prevent changes behavioural expression being triggered.

Therefore while these manipulations were not a success, there is potential to adjust the dose to one similar to that given to the weaned pups in their trials, which succeeded in effecting behavioural frequencies. If the IM route was used again, using a 2ml injection may be enough to penetrate central brain structures and trigger behavioural

changes rather than desensitise the oxytocin receptor network. If this dose was not sufficient to achieve a high enough plasma concentration for long enough duration to penetrate the BBB, then administering an IV dose could be tried to assess its feasibility with un-drugged mothers using only physical restraint to administer the injection. Further work is needed to determine the optimal dose for this age class of grey seal, but once an optimal dose is developed, we then have a tool to directly test the functional effects of biologically relevant high plasma oxytocin concentrations in mother grey seals, as was successfully done with the weaned pup study group earlier in this chapter.

Effect of intramuscular oxytocin manipulation

While in the one hour manipulation trials, mothers given the IM oxytocin injection had significantly elevated plasma concentrations for the entire time in the trials, and this manipulation did not persist more than three hours post trial. The manipulation raised the plasma oxytocin to a mean of 128.5pg/ml, which is much higher than concentrations found naturally in mothers with dependant pups, mothers experiencing peaks due to nursing behaviour (see Chapter 5.) and the 1 hour post-trial concentrations in manipulated weaned grey seal pups (described above). This indicates that the peripheral intravenous manipulation method and dose developed for this species in Chapter 6. was suitable to cause elevated oxytocin concentrations for the entire hour of the trial in mothers similar to the successful manipulations on weaned pups. However as discussed above, the extremely high plasma oxytocin concentrations may have desensitised the mother's oxytocin receptors (Robinson *et al.* 2003) and attenuated any behavioural responses natural oxytocin concentrations generate (Popik *et al.* 1992), leading to no changes in any behavioural frequencies in mother grey seals in these experiments unlike the successful newly weaned seal manipulations described in the first experiment of this chapter.

7.5 Conclusions

This study demonstrates that peripheral oxytocin injections in weaned grey seal pups elevate affiliative behaviours and reduce aggression, while manipulations in mothers with dependant pups did not change any measured frequency of behaviour. Weaned pups given IV oxytocin manipulations when encountering novel individuals decreased

their frequencies of investigative and aggressive behaviours towards each other, classic signs of social recognition, and additionally increased their frequency of approaching the other study animal and the time spent within one body length of each other. An order effect occurred during the weaned pup manipulations indicating the oxytocin injections may have triggered the formation of stable social memories of the other individual in the initial trial exposure to each other, however, further pen trials are needed to determine if weaned pups are naturally capable of quickly forming memories of other individuals. Finally while the mother manipulations did not trigger any behavioural changes across the two treatment groups, this may be due to too high a dose being administered to them, which could easily be rectified in future experiments.

Chapter 8: Social Recognition between Unrelated Grey Seals

8.1. Introduction

Individual recognition between conspecifics is a vital first step in linking past experiences to present companions during repeated interactions, allowing behaviours ranging from aggression to affiliation to be directed towards different individuals despite being part of the same group. Pinnipeds spend much of their lives foraging at sea without a stable social environment, however most species aggregate ashore in large numbers for short periods of time (Boness and Bowen 1996). These periods range from spending two to three weeks together to breed to a few hours together hauled out to rest or moult. Aspects of pinniped reproductive behaviour, such as breeding site fidelity and longevity, mean that individuals may encounter the same animals repeatedly within and across seasons and there is evidence to support the existence of fine scale social structure in some pinniped species (Pomeroy *et al.* 2005, Wolf *et al.* 2007, Lidgard *et al.* 2012). Therefore the potential for social bonds to form between adjacent animals in such seasonal aggregations exists in colonially breeding pinniped species.

The abilities of grey seals to recognise biologically unrelated animals and the potential mechanisms by which they do so have never been investigated. Published studies on male territorial behaviour provide evidence that otariids are capable of distinguishing adult conspecifics they have encountered before. Neighbouring rival male Antarctic fur seals (*Arctocephalus gazelle,* McCann 1980) and Australian fur seals (*Arctocephalus pusillus doriferus*, Tripovich *et al.* 2008) decrease the number of aggressive interactions and threat calls towards males with whom they have previously interacted or who occupy neighbouring territories. This disinterest in neighbouring rivals compared to novel individuals in male fur seals is attributed to the 'Dear Enemy' effect (Fisher 1954), which has been reported in species that utilise breeding territories in a wide variety of taxa including reptiles (Carazo *et al.* 2008), fish (Leiser 2003), birds (Briefer *et al.* 2007) and mammals (Rosell and Bjørkøyli 2002). For breeding grey seal mothers which fast during lactation (Boness and Bowen 1996), reducing aggressive interactions between adjacent females as seen in the 'Dear Enemy' effect would be beneficial in terms of reducing their energetic expenditure

towards behaviours that do not directly invest in their dependant pup. Female grey seals that heavily expend energetically in a pup have been shown to have lower breeding success in subsequent years (Pomeroy *et al.* 1999). If individuals recognise and adjust their behaviour towards one another, the risk of injury to themselves and dependent pups would be reduced. Fewer aggressive interactions may allow more energy to be directed towards reproductive output and selection for stable social bonds between adjacent mothers within the colony may occur. There is evidence that some breeding female grey seals change pupping locations in order to have their pup adjacent to females with whom they have previously associated during past breeding seasons, despite this species typically displaying strong site fidelity to one pupping site on a colony (Pomeroy *et al* 2005). However to date no study has investigated whether grey seals are actually capable of recognising other individual conspecifics. As this species has been shown to have variable ability in recognising their own pups from non-filial ones (McCulloch *et al* 1999, McCulloch and Boness 2000), it cannot be assumed that they can consistently recognise other biologically unrelated individuals.

Experiments investigating an individual's ability to recognise others rely upon changes in the frequency of specific behaviours between the trial animals. The most common of these is investigative behaviours, such as sniffing of the facial or anogential regions in rodents which typically decrease with familiarity (Bielsky and Young 2004). Classic paradigms for exploring social recognition, such as the social habituation/dishabituation paradigm, require that the primary trial animal is repeatedly exposed to another individual and any behavioural changes recorded. Finally the trial animal is exposed to a novel individual to confirm that the frequency of investigative behaviours return to a similar level to that seen at the start of the trial (Bielsky and Young 2004). In this chapter we use an experimental design similar to these rodent trials, adapted to allow for the differences between the focal species used (seals rather than rodents) to additionally test the hypothesis that familiar animals exhibit fewer aggressive behaviours towards each other.

While changes in behaviour provide evidence that a species is capable of recognition, this study will look for further evidence of this ability in the study animal's physiology. Oxytocin has been shown to be vital for the formation of social memory in mice (Ferguson *et al.* 2000 and 2001) and for linking interactions with unrelated familiar individuals to a physiological reward in humans and dogs (Odendaal and

Meintjes 2003). While it has been widely studied in human social interactions (Kosfeld *et al.* 2005, Guastella *et al.* 2008, Rimmele *et al.* 2009, Hurlemann *et al.* 2010) there are few studies looking at oxytocin's role in other wild vertebrate social systems (meerkats: Madden and Clutton-Brock 2011, chacma baboons: Moscovice and Ziegler 2012 and chimpanzees: Crockford *et al.* 2013). Oxytocin promotes social behaviour in two ways, first by acting on the amygdala to inhibit fearful or repulsive responses to the other individual (Petrovic *et al.* 2008) and second by activating reward and pleasure pathways to release neurotransmitters such as dopamine in the brain (Baskerville and Douglas 2010). Elevated levels of plasma oxytocin have previously been used to indicate positive social bonds in dogs (Odendaal and Meintjes 2003) and humans (Levine *et al* 2007, Feldman *et al* 2007) and therefore are an appropriate indicator of positive social experiences between unrelated individuals. This study investigates recognition abilities of grey seals under trial conditions using behavioural and endocrinological measures as indicators of familiarity. If seals are capable of discriminating between familiar and novel individuals, then the frequency of investigative and aggressive behaviours directed at each other is expected to fall when with familiar study subjects. Additionally we will investigate oxytocin's role in recognition between unrelated individuals through measurement of naturally occurring plasma concentrations during stranger/familiar encounters.

The objectives of this research were to:

1. Document the natural recognition abilities of grey seals using changes in behavioural frequencies as indicators of recognition between conspecifics.
2. To determine if plasma oxytocin concentrations are correlated to behavioural changes recorded during the trials.

8.2. Methods

8.2.1. Study site

This study took place on the Isle of May grey seal breeding colony in Scotland. Trials to study natural recognition abilities occurred during the 2010 season, from 12th November to 6th December.

8.2.2. Study animals and selection criteria for trials

Two rounds of pen trials were conducted in this study. Firstly a pilot trial using eight newly weaned grey seal pups penned in two groups of four was conducted for four days prior to the main trial. After this, a group of 12 weaned grey seal pups were penned in two groups of six for 14 days. Adult seals were not used in this component of the study due to the practical difficulties associated with their large size and aggressive temperament when approached by researchers. All study animals were weaned within four days of each other and the sex ratio in each pen was even. Pups were defined as being weaned on the second consecutive day of being seen without their mother based on daily observations.

Weaned pups were deemed a suitable substitute for adults as the oxytocin receptor system in brain regions associated with social behaviours are mature at weaning in rodents (Wang and Young 1997). Upon capture all pups were sexed, weighed and had a basal blood sample drawn using the methodology described in Chapter 2. Pups were weighed ±0.2 kg on a spring balance (Salter Industrial Measurements Ltd.,West Bromwich, UK). Individuals less than 30 kg were excluded from the study and early release criteria were set as a drop below 30 kg or 75% of their capture mass, in accordance with previous studies using captive weaned grey seal pups to prevent the post-weaning fast period from being extended to an unnatural length (Bennett *et al.* 2007). To assist in identification all animals were marked with gloss paint on the mid-dorsal region, receiving either a single letter or number depending on the pen in which they were held. Mass measurements were taken every three days throughout the period of captivity and none of the study animals lost sufficient weight to warrant early release.

8.2.3. Paired pen trial protocol

The two temporary holding pens were approximately 15 x 10 meters each and contained pools of fresh water. The trial pen where all experiments took place was a separate 5x5 meter structure out of sight from the holding pens. These were constructed with the permission of Scottish National Heritage and in accordance with UK Home Office guidelines on suitable temporary holding facilities for grey seals,

and were taken down once the trials were complete. Holding pens were located in a part of the island the breeding seals did not use. Individuals in one pen had never come into contact with the individuals in the other, and additionally animals within each pen had no prior experience of each other. This was verified by observations on the pups while they were still with their mothers at various sites scattered throughout the Isle of May breeding colony. The pens were separated by 10 meters to ensure that no physical interactions could occur through the fencing outside of the recognition trials (see Chapter 7.).

All study pups were allowed one rest day after capture to acclimatise to the holding pens and the five other individuals with them. Paired pen trials then commenced, testing either 'stranger' pairs using one animal from each pen or 'familiar' pairs using two animals from the same pen (Figure 8-1). Stranger trials used animals that had never encountered each other before while familiar trials used animals that occupied the same pen. The two subjects required for a trial were captured in the holding pens and then carried while restrained in a bag to the trial pen at the same time. Mean time spent capturing and transferring pups from the holding pen to the trial pen was 2.4 minutes (SD = 1.1 minutes). The animals were introduced into the trial pen simultaneously in a standardised manner and individual behaviours were observed and recorded from a hide in real time for one hour immediately following introduction to the pens (see Chapter 7.). Video footage was also taken to allow further decoding at a later date (video camera used: Panasonic HDC-TM60 full HD 1920x1080). After the trial the animals were returned to the holding pen from which they had originated. Six trials were run per day with all study pups being used once per day. After a subset of both types of trials (trials sampled: n=6, giving individuals sampled: n=12) plasma samples were taken immediately after the trial but before the pups were returned to the holding pens to investigate post trial oxytocin concentrations, and were taken as described in Chapter 2. No individual was used in more than one trial per day and there was a rest day between each trial day. In the 14 day period of captivity 40 paired pen trials took place, consisting of 20 stranger trials and 20 familiar trials.

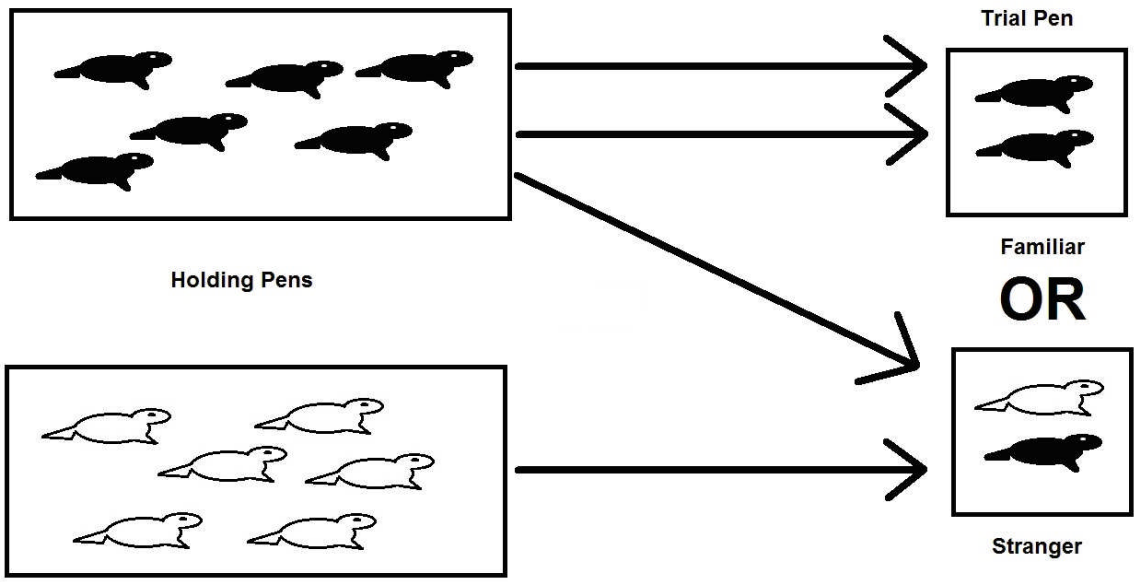


Figure 8-1. Paired pen trial design, with each holding pen containing six weaned seals and individuals from either the same pen (familiar trials) or from different pens (stranger trials) being used in the experiment.

8.2.4. Plasma sampling and analysis

In addition to post trial samples, plasma samples were taken at initial capture, midway through the captivity period, post release and after a subset of stranger and familiar trials. The initial capture, midpoint and post release samples were taken to ensure that being in a captive environment was not affecting basal oxytocin levels. Samples were collected using the capture, restraint and draw methodology outlined in Chapter 2. At each sampling event two vacutainers of plasma were taken typically. Plasma samples from free ranging weaned pups on the North Rona colony in 2009 (n=8), 2010 (n=13) and 2011 (n=15) and the Isle of May in 2010 (n=12) and 2011 (n=28) were also used for comparative purposes. Samples were stored and plasma was then analysed for oxytocin using the laboratory methodology outlined in Chapter 2. Inter-assay coefficient of variance over the nine plates used in this study was 5.7%.

8.2.5. Behavioural decoding

The key behaviours in this study were approaches, investigative and aggressive ones between the trial animals. All distances are measured in weaned pup lengths. The same ethogram that was used in the pen trials in Chapter 7. was used for these pen trials. Video footage of all trials was analysed to record the frequency of the behaviours in the following ethogram;

Approach behaviour

1. Approach – Distinctive locomotion towards the other trial animal, ending either within one body length of each other or involving travel over two body lengths towards the other individual.

Investigative behaviour

1. Visual check – subject raises head above ground and makes a definite movement to look specifically at the other trial subject or pup.
2. Olfactory check – subject approaches other study pup and extends the snout towards the other's face, anogential region or any other part of the body with nares open and vibrissae flaring. Physical contact with the other individual may or may not occur.

Aggressive behaviour

1. Open Mouth Threat – head held low but above the ground, neck extended, mouth open. May be accompanied by other aggressive behaviours (see below).
2. Vocalisation – any hissing, growling or howls uttered while interacting with the other trial animal.
3. Flippering – one or both fore flippers waved in a frantic manner at the other animal, may or may not come into contact.
4. Lunge – Attempt to bite the other individual, neck extends rapidly and then retracts without contact with the other study pup.
5. Bite – Actual physical contact of one animal's mouth to any part of the other animal's body, in a rapid and aggressive manner.

6. Startle – Visible, physical startle response to one pup approaching, touching or vocalising at the other trial animal. Typically a violent jumping or flinching action.
7. Flee – Rapid locomotion away from the other trial animal, resulting in separation of more than one body length between the two individuals.

Actions fitting these categories were tallied for all trial animals, giving a score for the number of 'checks' (investigative behaviours), approaches and aggressive behaviours per individual, per trial. The amount of time the trial animals spent within one weaned pup body length of each other was recorded. Colonially breeding grey seals typically maintain distances of 2 adult body lengths between themselves and neighbours (Pomeroy *et al*. 1994 and Twiss *et al*. 2000) therefore we assume any individual within one body length of another would be sufficiently close to evoke a response from the other animal.

8.2.6. Statistical analysis

Basal oxytocin levels were calculated for free roaming weaned pups from the Isle of May and North Rona colonies were compared by a one-way ANOVA to the study group's oxytocin levels at capture to check that a physiologically unusual group had not been accidentally selected for these trials. The data was analysed after a square root transformation since the original data were not normally distributed when submitted to a Shapiro Wilk test ($p=0.007$). For samples taken after recognition trials, any effect of trial type on oxytocin concentrations was analysed using a two-way ANOVA with trial type and sex as explanatory variables. Repeated measures ANOVA were performed on the longitudinal basal oxytocin measurements taken from the study pups throughout the period of captivity to detect any changes caused by captivity.

Four generalised additive mixed models (GAMs) (Wood 2006a) were used to analyse the behavioural tally data counting the frequency of approaches, investigative and aggressive behaviours and time spent in within one body length of each other. Biologically plausible variables included in the selection process for these models were; the sex of the focal individual, time spent in captivity and the behavioural frequencies recorded that were not the dependant variable in the current model

(approaches, investigations, aggression and time spent within one body length) (see Appendix C, Table C-1). The identities of both individuals in the trials were fitted as two random effects smooth (focal and response animal) (Wood 2006b) to control for pseudo-replication in the dataset from using the same individuals over multiple trials. Three of these models were fitted with Poisson error distributions with log links using the multiple generalized cross validation library mgcv (Wood 2006a) due to the presence of zero values in the data, while the model concerning time spent in one body length was fitted with a normal distribution. Model selection used backwards stepwise elimination through examination of R^2 values, QQ and residual plots to identify the best model for the data.

8.3. Results

8.3.1. Behavioural frequencies and trial type

For both investigation behaviours and aggressive interactions between trial animals, the GAM models showed that the frequency of both was significantly higher during stranger trials compared to familiar trials (mean increase: 19.2 investigations/trial, $p<0.001$ and 10.7 aggressive interactions/trial, $p<0.001$) (Table 8-1 and Figures 8-2 and 8-3). Sex was not a significant explanatory variable in either model and therefore it was eliminated during the model selection process. Time in captivity and the individual identities of the trial animals involved were also significant variables in both models ($p<0.001$ for both). There was no significant difference in the number of approaches or time spent within one body length of each other based on trial type. The only explanatory variable that impacted on these two variables was the identity of the other individual in the trial ($p<0.001$ for approaches and $p<0.05$ for time spent in one body length) (Tables 8-2, 8-3 and Appendix C, Tables C-2 and C-3).

Table 8-1. Mean values for the frequencies of approaches, investigative behaviour, aggressive behaviour and the time spent within one body length of the response animal for each type of trial with standard errors.

Trial type	Approaches	Investigative behaviours	Aggressive behaviours	Time within one body length (hours:minutes:seconds)
Familiar	6.2 (±0.17)	61.7 (±0.12)	18.3 (±0.29)	00:36:50 (±00:03:13)
Stranger	8.5 (±0.08)	80.9 (±0.03)	29 (±0.04)	00:38:53 (±00:02:11)

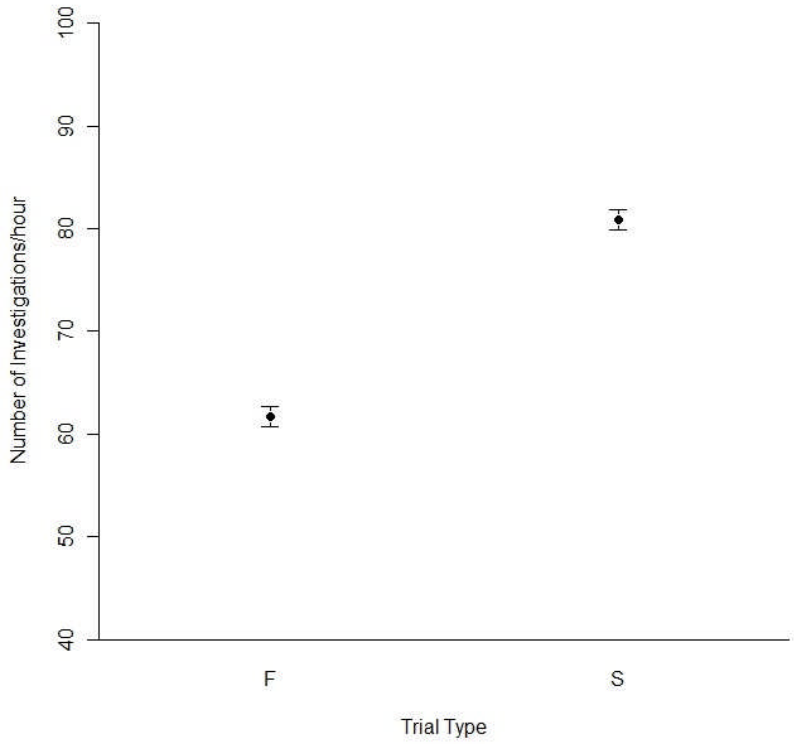


Figure 8-2. Mean frequency of investigative behaviours across the two trial types, familiar (F) and stranger (S) with standard error bars.

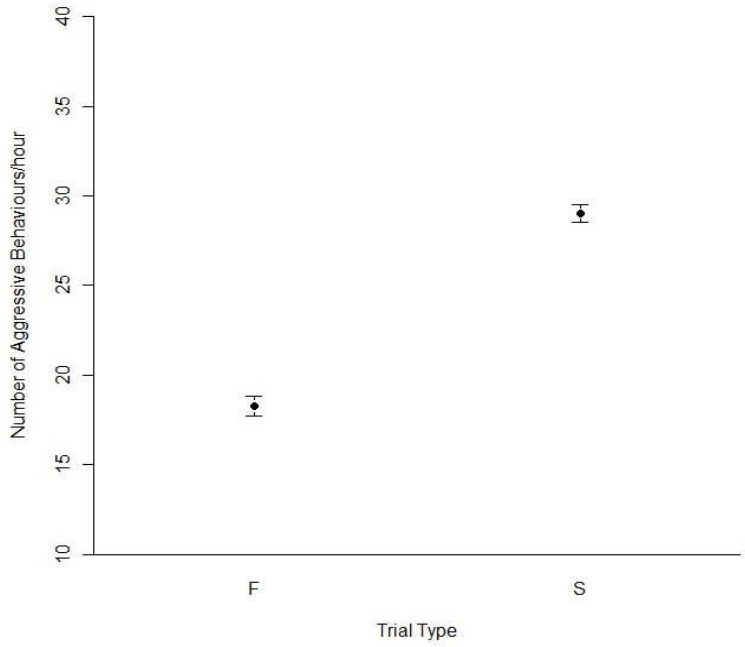


Figure 8-3. Mean frequency of aggressive behaviours across the two trial types, familiar (F) and stranger (S) with standard error bars.

Table 8-2. Significant fixed effect variables from the two GAMs concerned with the number of investigative behaviours and aggressive behaviours and their standard errors, estimates and p values.

Dependant variable	**Explanatory variable**	**Estimate**	**Standard Error**	**P value**
Investigations	Trial type (stranger)	0.09	0.03	<0.001
	Number of approaches	0.04	0.002	<0.001
	Number of aggressive interactions	0.004	0.0005	<0.001
	Time spent within 1 body length	0.0002	0.00003	<0.001
	Time spent in captivity	-0.02	0.004	<0.001
Aggressive interactions	Trial type (stranger)	0.4	0.05	<0.001
	Number of approaches	-0.05	0.007	<0.001
	Number of investigations	0.01	0.0009	<0.001
	Time spent within 1 body length	0.0003	0.00006	<0.001
	Time spent in captivity	-0.02	0.007	0.005

Table 8-3. Random effects from the two GAMs with significant differences across the two trial types and their standard deviations and p values.

Dependant variable	**GAMM random effect**	**Standard deviation**	**P value**
Investigations	Focal Individual	0.33	<0.001
	Response Individual	0.25	<0.001
Aggressive Interactions	Focal Individual	0.62	<0.001
	Response Individual	0.96	<0.001

8.3.2. Oxytocin concentrations and trial type

There were no significant differences in plasma oxytocin concentrations with trial type (stranger: 11.8 ±0.9 pg/ml, familiar: 11.1 ±0.9 pg/ml) ($F_{(1, 48)}$=0.46, p=0.5) or sex ($F_{(1, 48)}$=3.17, p=0.08).

8.3.3. Basal oxytocin detection

To compare the basal oxytocin level present in the 2010 study group to that of free roaming animals, a one way ANOVA was conducted. The test indicated there were significant differences between groups of weaned pups caught on different colonies in different years ($F_{6, 106}$ = 10.9, p<0.001) and therefore post hoc analysis was done using a Tukey honest significant differences test. There were no significant differences between the study group individuals (mean = 14.4 ±1.5 pg/ml) and the free roaming individuals from the Isle of May in 2010 (mean = 13.8 ± 1.5 pg/ml, p=0.9). However there were significant differences between some free-ranging groups in some sampling years and across the two colonies, including significant differences between the study group for pen trials conducted in 2011 (mean=7.5pg/ml, Chapter 7.) and the study group in 2010 (p<0.001) (please see Chapter 7. for full reporting and discussion of these results)

8.3.4. Oxytocin and captivity

A repeated measures ANOVA was performed to determine whether plasma oxytocin concentrations changed with increasing time in captivity. The time spent in captivity had no significant effect on basal plasma oxytocin in the study animals ($F_{2, 21} = 2.319$, $p=0.12$) (Figure 8-4).

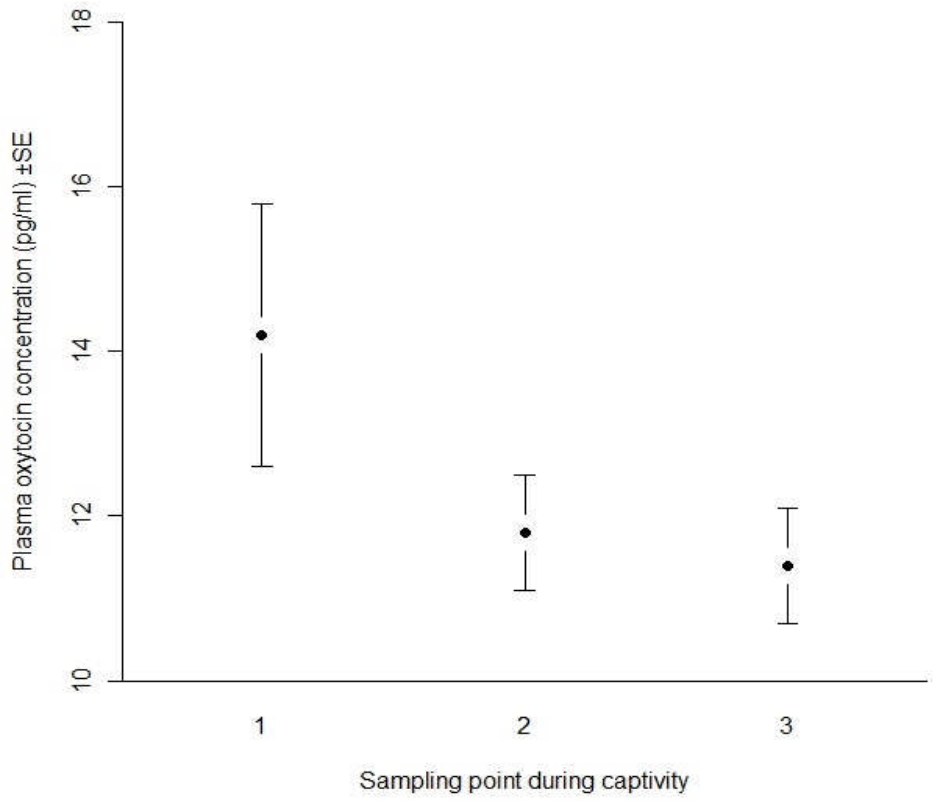


Figure 8-4. Mean plasma oxytocin (pg/ml) in the captive study group throughout their period of captivity, with the initial capture samples prior to entering the captive environment (1), mid point samples at day seven of captivity (2) and post release samples collected two days after release from a total of 14 days captivity (3) with standard error bars.

8.4. Discussion

8.4.1. Behavioural frequencies and trial type

This study detected differences in investigative and aggressive behaviour which weaned grey seal pups express towards familiar animals over novel, unfamiliar ones. This evidence suggests that grey seals are capable of recognising animals they have previously been exposed to, and adjust behavioural frequencies directed towards them by lowering the amounts of aggression.

Female grey seals face a range of challenges within a breeding colony, and energetic output via aggressive behaviour towards one's neighbours is a necessity of living in a dense colony environment. Selective pressure from aggressive males is thought to be the biggest factor in driving pinniped colony size upward (Cassini 1999, Camagna *et al.* 1992). However, the protection from males a breeding female obtains when surrounded by other mothers is offset by heightened aggression between each other (Redman 2002). Mothers have limited resources to utilise while fasting on a colony, and while fasting and nursing a pup, females can loose up to 40% of their body mass (mean mass loss ~80kg). If a substantial energetic investment is made during one breeding season, the individual's reproductive success will be negatively affected in the following years (Pomeroy *et al*. 1999). Therefore the optimal strategy for expressing aggressive behaviour on a breeding colony would be to recognise those that pose a real threat to the mother-pup pair and only direct aggression towards them. At the same time, an individual should habituate to neighbours that are regularly encountered who do not harass or injure the mother-pup pair. This would prevent wasting limited resources on unnecessary aggression and reduce the risk of injury to the mother or her pup.

These trials are the first to test directly for individual recognition between unrelated grey seals. Prior to this work, studies have only been able to predict the existence of recognition and stable social bonds on grey seal colonies from looking at factors governing female pupping site choices (Pomeroy *et al.* 2000, Redman *et al.* 2001) or as possible explanations of unexpected inter-annual patterns of affiliation (Pomeroy *et al.* 2005). The opportunity for stable social bonds to form between breeding females exists because they are long lived and exhibit site fidelity across breeding seasons

(Pomeroy *et al.* 2000) and also show little variation in the date they give birth every year (Pomeroy *et al.* 1994). These characteristics of female pupping behaviour mean that females return in successive seasons to pup adjacent to many of the same individuals as before, giving them repeated chances to interact with each other. Additionally, in females not exhibiting site fidelity in pupping location, a high proportion of them gave birth next to females they had previously associated with (Pomeroy *et al.* 2005). While these aspects of colony behaviour support the existence of recognition, none provide direct evidence for the ability to do so.

Repeat exposure trials in pen environments have been used to test for social recognition in rodents (Ferguson *et al.* 2000, Bielsky and Young 2004) and the study design has proved successful when using newly weaned seal pups. While it would have been preferable to use adult females to be easily comparable with animals on the breeding colonies, a number of factors prevented this from occurring. The large mass of the animals at adult size, and their aversion to people when on land make handling wild seals problematic. Constructing pens to successfully contain a group of adult animals would also have been extremely difficult, as they are strong enough to knock down barriers made of stone (S Moss, pers. comm) and mobile enough to climb fences or walls at least 2 metres high (KJ Robinson, pers. obs). The use of newly weaned animals instead of adults allowed us to take advantage of the post weaning fast this species exhibits for 1-4 weeks (Reilly 1991). Weaned pups can be captured, handled, transported and penned at this age, all crucial requirements for this study. Wang and Young (1997) showed that rats at weaning have mature oxytocin receptor distributions in regions of the brain concerned with social behaviour, such as the lateral septum. Changes in oxytocin receptor distributions in regions such as the bed nucleus of the stria terminalis are known to occur with puberty, but these are concerned with reproduction and parental behaviour which is not the focus of this study. An attempt to study the effect of oxytocin manipulations on mothers in small sub groups on the colony was made during the 2011 season of the Isle of May study. This would have allowed us to examine whether oxytocin manipulations stimulate different frequencies of behaviour towards their neighbours. However while attempting to perform these manipulations, an unacceptable amount of disruption to the breeding colony occurred and the doses of oxytocin and saline could not be consistently and accurately administered rapidly across multiple individuals. These trials therefore had to be discontinued, preventing us from investigating whether

oxytocin is responsible for changing frequencies of behaviour between adult unrelated individuals as it is in weaned pups (see Chapter 7.).

Our results showed that there were fewer investigative behaviours and aggressive interactions when individuals were in a trial with a familiar animal. In a pen trial setup this indicates that the individuals are recognising each other (Bielsky and Young 2004). The decline in both visual and olfactory checking is also seen in rodents undergoing pen trials with familiar conspecifics (Ferguson *et al.* 2000), and indicates that the animals are aware they have previously investigated the other individual and do not need to conduct further examinations. Reduction of aggression towards familiar individuals is often cited as a crucial step towards sociality (Kleiman and Eisenberg 1973, Barrett *et al* 2002, Estevez *et al* 2003). This result provides a reason why seals retain recognition abilities despite leading mostly solitary lives outside of colony environments. If individuals that recognise each other are capable of adapting the frequency of costly interactions between them, and this leads to increases in maternal investment and reproductive output, then recognition abilities will be selected for in the colony.

8.4.2. Oxytocin concentrations and trial type

None of the pups with novel or familiar companions showed any significant rise from basal oxytocin concentrations. Therefore while there is a significant behavioural difference in responses to novel individuals and those previously encountered, there is no evidence that oxytocin is playing a role in driving this change. One explanation for this may stem from oxytocin only having a role in positive social interactions, especially between individuals with an existing social bond (Uvnäs-Moberg 1998, Odendaal and Meintjes 2003, Nagasawa *et al.* 2009, Crockford *et al.* 2013). While all the pups had co-habited with each other for the same length of time, there was no way to control for how much time they spent familiarising themselves with the other pen occupants, or whether the outcome would be a positive bond between the two or a negative rejection.

As grey seals are not thought to spend the majority of their lives in social groups, there may be a good reason why behavioural plasticity towards unrelated individuals is not dependant on or influenced by central or peripheral oxytocin concentrations. Oxytocin acts as part of a conditioned positive feedback loop, where it is released

during positive social interactions to encourage further interaction with that individual (Uvnäs-Moberg 1998). If oxytocin release occurs, then it encourages the formation of stable pairs or groups that are then maintained for a period of time after the individual's first encounter. This has obvious benefits for maintaining groups in highly social species that rely on group living for their survival. However if this were occurring between unrelated individuals in a seal colony, then we would expect to see some expression of this behaviour throughout the breeding season and extending into the time post-breeding at sea. When the time comes to leave the colony, there is no evidence to indicate that seals maintain long term associations while foraging across vast distances at sea. Therefore oxytocin release during interactions between unrelated individuals would be inappropriate, and would encourage the expression of sub-optimal social behaviour that is not typical of this species or necessary for an individual's survival outside of the breeding season.

This indicates that the recognition abilities and changes to aggression frequencies between familiar individuals in grey seals are driven by neurological functions or hormones other than oxytocin. Phocid seals require a recognition system that allows recognition and reduction of unnecessary aggression without stimulating improper attachment to neighbours that may hinder separation at the end of the breeding season. Therefore there is no reason for a hormone that would reward and perpetuate bond-partner preferences to be released in unrelated conspecific interactions. There are other examples of this occurring in largely solitary species, such as black bears reducing aggression towards each other on human dump sites that provide a huge valuable food resource (Herrero 1983). These results additionally follow the 'Dear Enemy' phenomenon where individuals with adjacent territories reduce aggression towards each other over time and repeated interactions (Fisher 1954). However in our results familiar individuals additionally performed less investigative behaviour, which has been identified as a positive indicator of individual recognition abilities in rodents (Bielsky and Young 2004) and can be distinguished from habituation with the introduction of a novel individual returning investigative and aggressive behavioural frequencies to the initial level expressed during the first encounter.

Such social plasticity is needed in a species that must occupy the two extremes of the social range, going from solitary to living in an aggregation of hundreds of individuals, and for non-reproducing individuals to express oxytocin release to each others presence would perhaps be unnecessary for this species. Therefore it appears

that oxytocin only has a role in the mother/pup bond and the behaviour between those two individuals. Elevated concentrations that naturally occur during the breeding season may additionally affect a mother's behaviour as discussed in Chapter 5, however grey seals naturally posses the ability to modify their behavioural output towards familiar individuals without the elevation of this hormone.

8.4.3. Basal Oxytocin Concentrations

There was no difference between the basal oxytocin levels of the captive group and free-roaming weaned pups on the Isle of May in 2010, indicating that we had not sub-sampled an unusual and unrepresentative group from the study. However there were differences between some basal datasets from different years of sampling and different colonies. The same basal dataset of weaned pup plasma was used in Chapter 7. to test for abnormal study group sampling, please refer there for the discussion of this result.

8.4.4. Oxytocin and Captivity

While in captivity, pre-capture, mid-point and post capture oxytocin levels in the seals were all comparable. This indicates that along with sub sampling a representative group of individuals for the trials, the penning and study protocol did not impact on them to significantly alter their endocrinological states. As oxytocin is involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) stress responses (Tops *et al.* 2012), any stress caused by the pen environment and study protocol had the potential to alter the basal endocrine profiles of the study animals over time, introducing an undesired variable into our experiment and results. However, there is no evidence to indicate that this occurred during these trials.

8.5. Conclusions

This study demonstrates that grey seals can recognise animals they have prior experience of. We have shown they are then capable of adjusting their behaviour towards such individuals, reducing the number of potentially costly aggressive interactions they exchange. We have also found that in unrelated, weaned pups this process is independent of elevated peripheral oxytocin levels, unlike those found in mother-pup pairs. The attempt to uncover the effects of oxytocin on free ranging mothers proved unsuccessful and deprives us of the opportunity to discover more about the mechanisms of peripheral oxytocin and how it affects expressed behaviour in breeding adults. However this study provides a vital first step in understanding how the hormones tied to breeding and maternal behaviour may or may not also extend their influence into encouraging sociality among solitary creatures.

Chapter 9: General Discussion

9.1. Thesis Summary

Oxytocin has been extensively used to investigate the physiological factors driving maternal and social behaviour in human and domestic or laboratory animals. In this thesis, it has been utilised successfully for the first time to analyse the behavioural endocrinology of the maternal behaviour of wild mammals. An accurate, reliable analysis technique to detect the neuropeptide hormone in two substrates (plasma and milk) was validated for samples collected from three species of phocid seal (grey, harbour and Weddell) (Chapter 3. and Chapter 5.). Using this technique, samples collected from mothers and their dependant pups from two breeding colonies of grey seals in Scotland (North Rona and the Isle of May) were analysed alongside detailed behavioural observations from the pairs (Chapter 4.).

Plasma oxytocin concentrations were positively correlated with behaviours that made mother – pup separation less likely, such as the time spent in close proximity to each other, the number of checks a mother performed on her pup and the activity levels of the pup. Plasma oxytocin concentrations in mothers showing maladaptive maternal behaviours such as abandonment or pup theft were significantly lower than mothers that successfully raised their pups to weaning and were no different to concentrations found in non-breeding individuals (Chapter 5.).

To directly link plasma oxytocin concentrations to the expression or suppression of behaviour towards another individual, it was necessary to conduct a series of manipulation experiments. Firstly the methodology for elevating the plasma oxytocin concentration of free ranging seals was successfully developed (Chapter 6.). Manipulation trials using weaned seals then demonstrated that individuals with elevated plasma oxytocin showed less investigative and aggressive behaviours toward each other, approached more and spent more time in close proximity to one another. The manipulations on grey seal mothers, while successful physiologically, did not have an impact on the behaviour exhibited towards their pups (Chapter 7.).

Finally the ability of grey seals to recognise unrelated conspecifics on a colony and oxytocin's role in this process were investigated (Chapter 8.). Newly weaned grey seals are capable of recognising individuals they have co-habited with before, and

they reduce the frequency of aggressive behaviour they exhibit towards each other when compared with interactions between novel individuals. This change in behaviour was not accompanied by changes in basal oxytocin, suggesting that interactions between familiar individuals in grey seals are not reinforced by neuropeptide release centrally or peripherally as in mammals such as primates that live in social groups.

9.2. Methodologies for Analysing Oxytocin

Given the huge variation and controversy in published methodologies on analysing oxytocin in biological substrates (Szeto *et al.* 2011, McCullough *et. al* 2013) and the absence of any previous studies on wild mammal plasma oxytocin concentrations, validating a sample collection protocol and assay for use in this thesis was essential. In doing so, previously unknown sources of protocol induced variation in the published analysis methodology were uncovered (Chapter 3.). These potential sources of error are applicable to studies analysing this hormone in any species for any study using plasma as a substrate, whether investigating psychological, medical or behavioural endocrinology questions. Peripheral oxytocin concentrations are now regularly used in scientific research to investigate human psychological conditions, their causes and potential treatments. Conditions that have thus far been studied include post-partum depression (Levine *et al.* 2007, Feldman *et al.* 2010a, Skrundz *et al*. 2011), anxiety (Hoge *et al.* 2012, Weisamn *et al.* 2013a), autism (Modahl *et al.* 1998, Hollander *et al.* 2007, El-Masry *et al.* 2010, Dadds *et al.* 2013), depression (Scantamburlo *et al.* 2007, Holt-Lundstad *et al* 2011), stress (Heinrichs *et al.* 2003, Grippo *et al.* 2009), anorexia nervosa (Hoffman *et al.* 2012) and suicidal behaviour (Deisenhammer *et al.* 2012). Of the published studies analysing plasma oxytocin concentrations reviewed for this thesis, almost half use methodologies that this thesis has found to be inaccurate, calling into question the reliability of such research and whether the results and conclusions drawn from such studies are valid. There are still calls for more research into the validation of accurate assay techniques for plasma oxytocin (McCullough *et al.* 2013) as studies continue to be published using inaccurate methodologies despite several publications highlighting the problems with such methods (Cool and Debrosse 2003, Szeto *et al.* 2011). This thesis provides more evidence to support the use of the most accurate, while more expensive and time

consuming, methodologies while additionally highlighting previously unreported sources of error that a study may incur.

With the successful validation of the analysis methodology for samples taken from wild phocids for oxytocin research, there is great potential for similar validation to be carried out for other wild animal species and substrates. Both saliva (Carter *et al.* 2007, Feldman *et al.* 2010a and 2010b) and urine (Nagasawa *et al.* 2009, Moscovice and Ziegler 2012, Crockford *et al.* 2013) have been used to investigate social behaviour and oxytocin concentrations in humans and wild primates, however the accuracy of using these peripheral substrates to infer central processes have their critics (Horvat-Gordon *et al.* 2005). Therefore future research must be conducted into whether such substrates accurately reflect plasma or CSF concentrations of oxytocin, and over what timescale the detected concentrations change, if at all, when exposed to stimuli known to cause oxytocin release either centrally or peripherally. As both urine and saliva, or in the case of large cetaceans blow expirate, can be collected non-invasively without the need to capture and handle the individuals, there is much interest in whether such substrates can be used to accurately study oxytocin concentrations in both human and animal subjects. Only with rigorous investigation of potential source of methodological induced error, full validation of the detection protocols used and detailed documentation of the relationship between central, circulating and salivary or urinary oxytocin will researchers be able to confidently utilise such substrates to study behavioural endocrinology across the animal kingdom.

9.3. Methodologies for Manipulating Oxytocin

In Chapter 7, peripherally administered oxytocin doses where shown to have a significant effect on the behaviour an individual exhibits. This should, according to the majority of the literature on oxytocin, not be possible as the BBB is supposedly completely impenetrable to the hormone, allowing different concentrations to exist in the CSF and in peripheral circulation so that oxytocin can function as both a hormone and a neuropeptide (Ermisch *et al.* 1985a, Kang and Park 2000, Churchland and Winkielman 2012). There are a small but growing number of studies that have had success using peripheral oxytocin manipulations to cause central effects however (McCarthy 1990, Popik *et al.* 1992, Bales *et al.* 2007, Hollander *et al.* 2007, Grippo *et al.* 2009, Madden and Clutton-Brock 2011) and the work carried out in this thesis

supports the hypothesis that somehow, the change in peripheral oxytocin caused by the manipulation are triggering changes in the brain.

Oxytocin and oxytocin agonists have been proposed and in some cases trialled as clinical treatments for conditions ranging from those affecting social behaviour and anxiety such as autism (Hollander *et al.* 2007) and schizophrenia (Churchland and Winkielman 2012) to those affecting the basic physiology of the patient such as diabetes and cancer (Viero *et al.* 2010). Research into methods of successfully manipulating peripheral and central concentrations of the hormone, and understanding how the two systems interact, are therefore in demand in order to further develop therapeutic applications for the hormone (MacDonald and MacDonald 2010, Churchland and Winkielman 2012, Kagerbauer *et al.* 2013). This thesis has shown a dose dependant effect on the clearance rate of oxytocin manipulations, and hypothesised that this was a result of BBB penetration which triggered further release of the hormone into circulation (Chapter 6.). Future studies may therefore seek to validate or disprove this hypothesis using similar methodologies to those used in Mens *et al.*'s 1983 study that successfully measured the proportion of IV administered oxytocin that crossed the BBB and caused peaks in the CSF.

The success of using peripheral oxytocin manipulations on wild animal species to investigate the physiological processes driving their behaviour means that such studies in other wild animal species can be performed as long as it is practically possible and ethically viable to perform an IV or IM injection on individuals. Many studies on wild mammalian behavioural endocrinology, as with the majority of studies carried out on wild populations, are correlatory or descriptional (Romesburg 1981, Ratti and Garton 1994, Hardy *et al.* 2003). This is because of practical and ethical constraints making experimental research, such as manipulation trials, impossible. However, the manipulation methodology developed in this thesis would allow investigation of the affects of oxytocin in a wide range of mammalian species, both in captive or wild environments given the right experimental design and dose. As all mammals display some form of maternal behaviour, and complex social behaviour exists in many taxa, experiments looking to link the underlying physiology of a species with the behaviour an individual expresses, and the impact this has on reproductive success, individual survivorship and health could take place. Furthermore, as almost all vertebrate life forms produce oxytocin or an oxytocin analog (Gimpl and Fahrenholz 2001) and as manipulation experiments on both fish

(Oldfield and Hofmann 2011, Braida *et al.* 2012, Reddon *et al.* 2012) and birds (Goodson *et al.* 2009, Pedersen and Tomaszycki 2012) have been successfully conducted in laboratory settings, there is no reason why such methodologies should be restricted only to experimental designs involving mammals. Such manipulation experiments would provide a vital link to allow hypotheses generated in correlatory studies on free ranging, wild animals to be confirmed or rejected.

9.4. Oxytocin and Maternal Behaviour

This thesis provides the first explanation for the seemingly random failure of an otherwise successful mother to correctly express maternal behaviour during a reproductive event (Chapter 5.) outside of stochastic events that reduce her fitness physically or energetically at the time. Additionally, oxytocin was found to stimulate behaviours that increase the likelihood of a pup surviving to weaning age by reducing the probability that the mother-pup pair would become separated. This is the first study to link oxytocin concentrations in wild individuals to the maternal behaviour they exhibit and to the fitness benefits or reproductive success that occur as a consequence of the physiology underlying these behaviours.

Due to the nature of hormonal control of behaviour, virtually any behavioural trait relying on endocrine systems to stimulate an appropriate behavioural response is potentially vulnerable to occasional critical failures. Reproductive events requiring seasonal triggers or mass participation to succeed are obvious examples of occasions where a failure of the external stimuli to trigger the appropriate endocrinological and therefore, behavioural response would result in maladaptive behavioural expression in an individual. It has been suggested that behaviours such as migration (salmonids (*Oncorhynchus sp.),* Iwata 1995) and hibernation (chipmunks (*Tamias sibiricus,)* Kondo *et al.* 2006) have endocrinological systems that respond to environmental triggers to initiate and regulate their expression, with severe consequences for an individual if it fails to correctly perform such behaviours. In species that rely on mass mating or spawning strategies, such as some crustaceans (e.g. the mangrove crab *Ucides cordatus,* Schmidt *et al.* 2012) or some snakes (reviewed in Shine 2003), endogenous controls of such events have also been suggested. One potential analogous case to the maternal grey seal results presented here may be present in garter snakes (*Thamnophis sirtalis parietalis).* In this species, male snakes in mating

balls sometimes misdirect mating behaviour towards other males, and this was found to confer no advantage to the individuals involved (Shine *et al.* 2003). Male mating behaviour has been shown to be independent of their own gonadal activity or steroid hormones at the time of mating (Crews *et al.* 1984), but instead is governed by hormonal concentrations that occurred up to eight months ago during the summer in that individual (Crews 1991). This is a critical window in which an appropriate hormonal response must be initiated in order to drive successful reproductive behaviour. This is highly analogous to the one hour sensitive period for mother-pup bonding found in the grey seal mothers (Chapter 5.), because if a certain pattern of hormone expression does not occur at a precise time, behaviour crucial to the individual's reproductive success cannot occur during that reproductive event but has no impact on the success of future seasons. The results of this study only permit insights into oxytocin's expression in breeding mothers, but as oxytocin is a highly conserved peptide in the mammalian clade, and as almost all vertebrate organisms express some form of oxytocin-like peptide (Gimpl and Fahrenholz 2001), such problems with its consistency in reliably triggering appropriate forms of behaviour are unlikely to be constrained to only grey seals.

Investigating the prevalence of such critical failures in behavioural endocrinology so to distinguish them from species that may have evolved high frequencies of apparently maladaptive behaviour due to a lack of selection pressure against them would be an excellent extension of the current work. Species such as the Mediterranean monk seal have high incidences (>50% of females observed) of fostering and pup theft behaviours with no negative impact on pup survivorship (Cedenilla *et al* 2009). However, behavioural strategies that may have been successful in a large population or colony may not continue to be viable in a small or declining colony, which is the trend for the population of this critically endangered phocid, and such behaviour may put additional pressure on to a species in decline from other factors. Understanding the prevalence of behavioural strategies that cannot adapt to a changing environment due to physiological constraints, such as the finite bonding period post partum, may be crucial for understanding why a population is declining or failing to recover from sources of anthropomorphic disturbance, and this may be important for developing key management strategies for the preservation of the species.

Disruption to mother-infant pairs by humans has been directly linked to declines in maternal care (harp seals, Kovacs and Innes 1990) and is widely hypothesised to be a risk factor for populations subjected to increasing disruption from human activities by both scientists (Lair *et al.* 2013) and mitigators of anthropogenic disturbance to wildlife (Roger *et al.* 2010). To better understand why such disruption can occur, investigation of the key sensory modalities different species utilise to form mother-infant bonds would be essential. While the key sensory stimuli for humans (reviewed in Uvnás-Moberg 1998) and sheep (Lévy *et al.* 1995) have been established in the literature already, the same modalities are unlikely to be important for maternal bonding across all species. Oxytocin is released into circulation by a mother with a strong bond to her offspring when receiving stimuli from it (Strathearn *et al.* 2009). Therefore, experiments to test which sensory modalities trigger such release could be conducted on a variety of species in captivity. This would provide crucial knowledge of whether sight, sounds, scent, tactile contact with an infant or a combination of them are important in establishing the bond that will sustain the pair association through out the dependant period, or how likely human disturbances are to interfere with the bonding process.

9.5. Oxytocin and Social Behaviour

Oxytocin has been shown to affect many aspects of human social behaviour (Kosfeld *et al.* 2005, Guastella *et al.* 2008, Rimmele *et al.* 2009, Hurlemann *et al.* 2010), however in this thesis no link between individuals that recognise each other and oxytocin concentrations was conclusively found. In Chapter 8. the recognition abilities of newly weaned grey seals was investigated and even at this young age, individuals were able to recognise and change their behaviour towards individuals that they had previously encountered. Testing for recognition abilities in pinniped species had previously been limited to repeated interactions between competing, territorial males on breeding colonies (e.g. Australian fur seals, Tripovich *et al.* 2008) and this is the first study to examine pinniped recognition abilities outside of this setting. Studies hypothesising the existence of social structure in colonies of grey seal mothers based on repeated associations (Pomeroy *et al.* 2005) now have evidence that conspecifics are capable of recognising each other despite having variable abilities to recognise their own pups (McCulloch *et al* 1999, McCulloch and Boness 2000).

Future work investigating the duration of individual's memories and the speed in which they acquire them when confronted with a novel individual would be interesting to quantify for pinniped species. Individuals may have very brief periods of contact with another conspecific while hauled out resting around the coast, or may have several days or weeks to interact with one another if the same individuals return to the same haul out or if they originate from the same breeding colony. Grey seals have also been demonstrated to form short term associations while foraging at sea (Lidgard *et al.* 2012), and the stability of such associations or whether they result in persistent memories is completely unknown. By attempting to define the typical time span recognition abilities extend over and how quickly individuals can acquire them, both short term associations and repeated ones occurring year after year on breeding colonies can be better understood.

9.6. Oxytocin as an Endocrine Indicator of Maternal Quality and Stress

In this thesis, plasma oxytocin concentrations were used to indicate the presence or absence of strong bonds between mothers and pups (Chapter 5.). As the only currently existing methods used to test the strength of maternal attachment are designed for humans who are capable of answering questions (Strathearn *et al.* 2009), they are completely unsuitable to studies on either wild or captive animals. Measuring plasma oxytocin concentrations therefore provides the only way to attempt to quantify this psychological trait in animal species which, while being difficult to assess, is a crucial variable in the dependant period of mammals. Oxytocin may be a valuable proxy to analyse when investigating another important aspect of an individual's welfare that is difficult to measure, the amount of stress an individual is currently experiencing (acute stress) or has experienced over a certain time period (chronic stress).
There have been several studies that have attempted to define the relationship between oxytocin concentrations and the action of the hypothalamic-pituitary-adrenal axis (HPA), which mediates the bodies' response to stressors (Nelson 2000), and specifically its relationship with cortisol and adrenocorticotropic hormone (ACTH), both hormones that can be analysed as biomarkers for stress. However, thus far the existing literature has not precisely nor extensively documented the relationship between the hormones (Veiro *et al.* 2010) and some of the reported findings appear contradictory. Oxytocin manipulations have been shown to reduce the cortisol

response to acute stressors in sheep plasma (Cook 1997) and human saliva (Ditzen *et al.* 2009) and plasma oxytocin has been shown to have an inverse relationship to plasma cortisol and ACTH concentrations in humans (Legros *et al.* 1984). However cortisol manipulations have additionally been shown to increase plasma oxytocin concentrations in humans (Tops *et al.* 2012), and some studies report plasma oxytocin concentrations falling in response to stressors (rhesus monkeys, Kalin *et al.* 1985) while others report plasma oxytocin rising during acute (voles, Grippo *et al.* 2009) or long duration stressors (Pournajafi and Carter, unpublished observations). These findings clearly indicate that there is a significant relationship between cortisol and oxytocin, and therefore oxytocin may be a suitable hormone to analyse in addition to cortisol or potentially in its place, as it does not rapidly respond to handling stress (Chapter 3.) like cortisol (Harcourt *et al.* 2010). However further work to clarify the relationship between these hormones is needed before oxytocin could be accurately and meaningfully used as an indicator of physiological stress.

9.7. Conclusions

This thesis has demonstrated that using peripheral measures of the hormone oxytocin can provide novel, important insights into the physiological factors driving maternal behaviour in a wild animal species. In developing a sample collection and assay protocol, it has become apparent that despite its recent popularity in scientific literature, many existing studies investigating this hormone have failed to properly validate the analysis methodology they are using and contain inaccuracies in their results. With proper validation however, a variety of substrates, including those collected non-invasively, could be analysed for this hormone from any animal species.

Analysing oxytocin concentrations is a powerful tool to link behaviours that impact on lifetime reproductive success in an individual to its physiology, and ultimately to the underlying genes that regulate the production and release of the peptide. Understanding the limitations or plasticity a physiological system has is vital to understanding whether a behavioural response dependant on hormones can adapt to a changing environment or is constrained to particular responses due to endocrinological constraints. Such knowledge is crucial for understanding the persistence of apparently maladaptive behaviour in the repertoire of species in

contexts where strong selection pressure should eradicate it from the population. As many aspects of an individual's behaviour that are crucial to reproduction are regulated by hormone expression, it is unlikely that maternal bonding is the only form of behaviour that cannot adapt to overcome disruption due to endocrinological constraints.

The major consideration for future studies on oxytocin in any species is the type of substrates used, and how concentrations in the different biological mediums interact with each other, if at all. It has only recently been documented that there is no direct correlation between CSF and plasma concentrations of oxytocin in humans (Kagerbauer *et al.* 2013) despite many previously published studies depending on such a relationship to exist. However with the growing body of evidence supporting the permeability of the BBB to oxytocin, dose dependant relationships between plasma and CSF oxytocin may exist under certain circumstances. With so many contradictions and unanswered questions about the relationship between central and peripheral oxytocin concentrations, care must be taken when conducting any oxytocin study not to infer causality solely from correlatory studies linking observed oxytocin concentrations to frequencies of behaviours. With the manipulation methodology outlined in this thesis, it is possible to experimentally link elevated hormone concentrations to changes in the frequency of expressed behaviours. This coupled with correlatory studies on the behavioural endocrinology of individuals in the wild make a powerful investigative combination. Together they not only directly link hormones and behaviours, but show that similar relationships between the two exist under natural conditions and play an important role in the success or failure of a reproductive event. Such a research approach can be applied to a wide range of species to investigate not only maternal behaviour or the wide range of traits that oxytocin plays a role in regulating, but any hormone that is involved in the control of behavioural expression.

References

Alexander, G., Poindron, P., Le Neindre, P., Stevens, D., Lévy, F. and Bradley, L. (1986) Importance of the first hour post-partum for exclusive maternal bonding in sheep. *Applied Animal Behaviour Science vol. 16, No. 3, P295-300*

Amico, J.A., Seif, S.M. and Robinson, A.G. (1981) Oxytocin in human plasma: correlation with neurophysin and stimulation with estrogen. *The Journal of Clinical Endocrinology and Metabolism vol 52, No. 5, P988-993*

Amico, J.A., Mantella, R.C., Vollmer, R.R. and Li, X. (2004) Anxiety and stress responses in female oxytocin deficient mice. *Journal of Neuroendocrinology vol. 16, No. 4, P319-324*

Amos, B., Twiss, S., Pomeroy, P. and Anderson, S. (1995) Evidence for mate fidelity in the gray seal. *Science vol. 268, No. 5219, P1897-1899*

Anderson, S.S., Burton, R.W. and Summers C.F. (1975) Behaviour of grey seals (*Halichoerus grypus*) during a breeding season at North Rona. *Journal of Zoology vol 177, P179-195*

Anderson, S.S., Baker, J.R., Prime, J.H. and Baird, A. (1979) Mortality in grey seal pups: incidence and causes. *Journal of Zoology vol 189, P407-417*

Anderson, S.S. and Harwood, J. (1985) Time budgets and topography: how energy reserves and terrain determine the breeding behaviour of grey seals. *Animal Behaviour vol. 33, No. 4, P1343-1348*

Assali, N.S., Dasgupta, K., Kolin, A. and Holms, L. (1958) Measurement of uterine blood flow and uterine metabolism v. changes during spontaneous and induced labor in unanesthetized pregnant sheep and dogs. *American Journal of Physiology – Legacy Content vol 195, No. 3, P614-620*

Assay Designs (2010) Oxytocin EIA Kit, Product Data. *Assay Designs Inc, Michigan, USA http://www.assaydesigns.com/ccpa0-1164-1164-oxytocin-eia-kit.htm*

Atzil, S., Hendler, T. and Feldman, R. (2011) Sepcifying the neurobiological basis of human attachment: brain, hormones, and behaviour in synchronous and intrusive mothers. *Neuropsychopharmacology vol 36, P2603-2615*

Baker, J.R. (1984) Mortality and morbidity in grey seal pups (*Halichoerus grypus*). Studies on its causes, effects of environment, the nature and sources of

infectious agents and the immunological status of pups. *Journal of Zoology vol 203, No. 1, P23-48*

Baker, J.R. and Baker, R. (1988) Effects of environment on grey seal (*Halichoerus grypus*) pup mortality. Studies on the Isle of May. *Journal of Zoology vol 216, No. 3, P529-537*

Bale, T.L. and Dorsa, D.M. (1997) Cloning novel promoter sequence, and estrogen regulation of a rat oxytocin receptor gene. *Endocrinology vol 138, No. 3, P1151-1158*

Bales, K.L., Pfeifer, L.A. and Carter, C.S. (2004) Sex differences and developmental effects of manipulations of oxytocin on alloparenting and anxiety in prairie voles. *Developmental Psychobiology vol 44, No. 2, P123-131*

Bales, K.L., van Westerhuyzen, J.A., Lewis-Reese, A.D., Grotte, N.D., Lanter, J.A. and Carter, C.S. (2007) Oxytocin has dose-dependent developmental effects on pair-bonding and alloparental care in female prairie voles. *Hormones and Behavior, vol 52, no. 2, P274-279*

Barrett, L., Gaynor, D. and Henzi, S.P. (2002) A dynamic interaction between aggression and grooming reciprocity among female Chacma baboons. *Animal Behaviour vol. 63, No. 6, P1047-1053*

Baskerville, T.A. and Douglas, A.J. (2010) Dopamine and oxytocin interactions underlying behaviours: potential contributions to behavioural disorders. *CNS Neuroscience and Therapeutics vol 16, No. 3, P92-123*

Baumgartner, T., Heinrichs, M., Vonlanthen, A., Fischbacher, U. and Fehr, E. (2008) Oxytocin shapes the neural circuitry of trust and trust adaptation in humans. *Neuron vol 58, No. 4, P639-650*

Bennet, K.A., Speakman, J.R., Moss, S.E., Pomeroy, P. and Fedak, M.A. (2007) Effects of mass and body composition on fasting fuel utilisation in grey seal pups (*Halichoerus grypus* Fabricius): an experimental study using supplementary feeding. *The Journal of Experimental Biology vol 210, P3043-3053*

Bernstein, I.S. (1984) The adaptive value of maladaptive behaviour, or you've got to be stupid in order to be smart. *Ethology and Sociobiology vol 5, No. 4, P297-303*

Bielsky, I.F. and Young, L.J. (2004) Oxytocin, vasopressin, and social recognition in mammals. *Peptide, vol 25, No. 9, P1565-1574*

Boness, D.J. and Bowen, W.D. (1996) The evolution of maternal care in pinnipeds. *Bioscience vol 46, No. 9, P645-654*

Bonner, W.N. (1989) The Natural History of Seals. *Christopher Helm Publishers, Kent, UK.*

Bowen, W.D., Boness, D.J. and Oftedal, O.T. (1987) Mass transfer from mother to pup and subsequent mass loss be the weaned pup in the hooded seal, *Ciystophora cristata. Canadian Journal of Zoology vol 65, P1-8*

Boyd, J.M., Lockie, J.D. and Hewer, H.R. (1962) The breeding colony of grey seals on North Rona, 1959. *Proceedings of the Zoological Society of London vol 138, No. 2, P257-277*

Boyd J.M. and Campbell, R.N. (1971) The grey seal (Halichoerus grypus) at North Rona, 1959 to 1968. *Journal of Zoology vol 164, No. 4, P469-512*

Boyd, I.L, Lockyer, C and Marsh, H.D. (**1999)** Reproduction in marine mammals. *In: Reynolds, J.E. and Rommel, S.A. (eds.) Biology of Marine Mammals. Washington, D.C., Smithsonian Institute Press, 218-286*

Braida, D., Donzelli, A., Martucci, R., Capurro, V., Busnelli, M., Chini, B. And Sala, M. (2012) Neurohypophyseal hormones manipulation modulate social and anxiety-related behaviour in zebrafish. *Psychopharmacology vol 220, No. 2, P319-330*

Briefer, E., Aubin, T., Lehongre, K and Rybak, F. (2008) How to identify dear enemies: the group signature in the complex song of the skylark *Alauda arensis*. *The Journal of Experimental Biology vol 211, P317-326*

Brockie, K. (1984) One Man's Island: a Naturalist's Year. *Harper & Row, London, UK.*

Broad, K.D, Curley, J.P and Keverne, E.B (2006) Mother–infant bonding and the evolution of mammalian social relationships. *Philosophical Transactions of the Royal Society B: Biological Sciences vol 361, No. 1476, P2199-2214*

Burton, R. W., Anderson, S. S. and Summers, C. F. (1975) Perinatal activities in the grey seal (*Halichoerus grypus*). *Journal of Zoology vol 177, P197-201*

Camagna, C., Bisioli, C., Quintana, F., Perez, F. and Vila, A. (1992) Group breeding in sea lions: pups survive better in colonies. *Animal Behaviour vol 43, P541-548*

Carazo, P., Font, E. and Desfilis, E. (2008) Beyond 'nasty neighbours' and 'dear enemies'? Individual recognition by scent marks in a lizard (*Podarcis hispanica*). *Animal Behaviour vol 76, No. 6, P1953-1963*

Carter, C.S. (1998) Neuroendocrine perspectives on social attachment and love. *Psychoneuroendocrinology vol 23, No. 8, P779-818*

Carter, C.S. (2003) Developmental consequences of oxytocin. *Physiology and Behaviour vol 79, No. 3, P383-397*

Carter, C.S., Pournajafi-Nazarloo, H., Kramer, K. M., Ziegler, T.E., White-Traut, R., Bello, D. and Schwertz, D. (2007) Oxytocin: behavioral associations and potential as a salivary biomarker. *Annals of the New York Academy of Science vol 1098, P312-322*

Castellini, M.A. and Somero, G.N. (1981) Buffering capacity of vertebrate muscle: correlations with potentials for anaerobic function. *Journal of Comparative Physiology vol 143, No. 2, P191-198*

Caudron, A.K., Kondakov, A.A. and Siryanov, S.V. (1998) Acoustic structure an individual vaiation of grey seal (*Halichoerus grypus*) pup calls. *Journal of the Marine Biological Association of the United Kingdom vol 78, No. 2, P651-658*

Cassini, M.H. (1999) The evolution of reproductive systems in pinnipeds. *Behavioural Ecology vol. 10, No. 5, P612-616*

Cedenilla, M.A., Fernández de Larrinoa, P., Haye, M., Varea, A., M'Bareck, H., Maroto, A., González, L.M. and Munoz-Canas, M. (2009) Description of adoption behaviour detected in the mediterranean monk seal (*Monachus monachus*) at the colony of the Cabo Blanco Peninsula (Mauritania – Morocco). *Poster session presented at:* 23rd Annual European Cetacean Society Conference, 2009 2-4 March, Istanbul, Turkey.

Cho, M.M., DeVries, A.C., Williams, J.R. and Carter, C.S. (1999) The effects of oxytocin and vasopressin on partner preferences in male and female praire voles (*Microtus ochrogaster*). *Behavioural Neuroscience vol 113, No. 5, P1071-1079*

Chuang Y.J., Swanson R., Srikumar M., Raja M and Olson S.T. (2001) Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. *Journal of Biolological Chemistry vol 276, No. 18, P14961-14971*.

Churchland, P.S., and Winkielman, P. (2012) Modulating social behaviour with oxytocin: how does it work? What does it mean? *Hormones and Behaviour vol 61, No. 3, P392-399*

Clutton-Brock, T.H. and Harvey P.H. (1978) Mammals, resources and reproductive strategies. *Nature vol 273, P191-195*

Clutton-Brock, T.H. (1984) Reproductive effort and terminal investment in iteroparous animals. *The American Naturalist vol 123, No. 2, P212-229*

Collins, K.T., Rogers, T.L., terhune, J.M., McGreevy, P.H., Wheatley, K.E. and Harcourt, R.G. (2005) Individual variation of in-air female 'pup contact' calls in Weddell seals, *Leptonychotes weddellii*. *Behaviour vol 142, No. 2, P167-189*

Conradt, L., Clutton-Brock, T.H. and Guinness, F.E. (1999) The relationship between habitat choice and lifetime reproductive success in female red deer. *Oecologia vol 120, No. 2, P218-224*

Cook, C.J. (1997) Oxytocin and prolactin suppress cortisol responses to acute stress in both lactating and non-lactating sheep. *Journal of Dairy Research vol 64, No 3, P327-339*

Cool, D.R. and DeBrosse D. (2003) Extraction of oxytocin and arginine-vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption-ionization time-of-flight mass spectrometry. *Journal of Chromatography B vol 792, No. 2, P375-380*

Costa, D.P., Le Boeuf, B.J., Huntley, A.C. and Ortiz, C.L. (1986) The energetics of lactation in the Northern Elephant Seal, *Mirounga angustirostris*. *Journal of Zoology vol 209, P21-33*

Crews, D. Camazine, B., Diamond, M., Mason, R., Tokarz, R.R. and Garstka, W.R. (1984) Hormonal independence of courtship behaviour in the male garter snake. *Hormones and Behaviour vol 18, No. 1, P29-41*

Crews, D. (1991) Trans-seasonal action of androgen in the control of spring courtship behaviour in male red-sided garter snakes. *Proceedings of the National Academy of Science if the United States of America vol 88, No. 9, P3545-3548*

Crockford, C., Wittig, R.M., Langergraber, K., Ziegler, T.E., Zuberbühler, K. and Deschner, T. (2013) Urinary oxytocin and social bonding in related and unrelated wild chimpanzees. *Proceedings of the Royal Society B vol 280, 20122765*

Culloch, R.M. (2012) The application of modern statistical approaches to identify consistent individual differences in the behaviour of wild postpartum female grey seals (*Halichorus grypus*). *PhD thesis, Durham University, UK*

Cushing, B.S., Levine, K. and Cushing, N.L. (2005) Neonatal manipulation of oxytocin influences female reproductive behaviour and success. *Hormones and Behaviour vol 47, No. 1, P22-28*

Da Costa, A.P.C., Guevara-Guzman, R.G., Ohkura, S., Goode J.A. and Kendrick, K.M. (1996) The role of oxytocin release in the paraventricular nucleus in the control of maternal behaviour in the sheep. *Journal of Neuroendocrinology vol 8, No. 3, P163-177*

Dadds, M.R., MacDonald, E., Cauchi A., Williams, K., Levy, F. and Brennan, J. (2013) Nasal oxytocinfor social deficits in childhood autism: a randomized controlled trial. *Journal of Autism and Developmental Disorders, P1-11*

Dale, H.H. (1906) On some physiological actions of ergot. *Journal of Physiology vol 34, No. 3, P163-206*

Darling, F. (1939) A Naturalist on Rona. *Clarendon Press, Oxford, UK.*

Darwin, C. (1859) On the Origin of Species by means of Natural Selection. *John Murray, London, UK*

Dawood, M.Y., Lauersen, N.H., Trivedi, D., Ylikorkala, O. and Fuchs, F. (1979) Studies on oxytocin in the baboon during pregnancy and delivery. *Acta Endocrinologica vol 91. No. 4, P704-718*

Dawood, M.Y., Ylikorkala, O. and Fuchs, F. (1980) Plasma oxytocin levels and disappearance rate after buccal Pitocin. *American Journal of Obstetrics and Gynecology vol 138, No.1, P20*

Dawood, M.Y., Khan-Dawood, S., Wahi, R.S. and Fuchs, F. (1981) Oxytocin release and plasma anterior pituitary and gonadal hormones in women during lactation. *Journal of Clinical Endocrinology and Metabolism vol 52, No. 4, P678-683*

DeGabriel, J.L., Moore, B.D., Foley, W.J. and Johnson, C.N. (2009) The effect of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. *Ecology vol 90, No. 3, P711-719*

Deisenhammer, E.A., Hofer, S., Scwitzer, O., Defrancesco, M., Lemmler, G., Wildt, L. and Hinterhuber, H. (2012) Oxytocin plasma levels in psychiatric

patients with and without recent suicide attempt. *Psychiatry Research vol 200, No. 1, P59-62*

Devarajan, K. and Rusak, B. (2004) Oxytocin levels in the plasma and cerebrospinal fluid of male rats: effects of circadian phase, light and stress. *Neuroscience Letters vol 367, No. 2, P 144-147*

Ditzen, B., Schaer, M., Gabriel, B., Bodenmann, G., Ehlert, U. and Heinrichs M. (2009) Intranasal oxytocin increases positive communication and reduces cortisol levels during couple conflict. *Biological Psychiatry vol 65. No. 9, P728-731*

Dluzen, D.E., Muraoka, S., Engelmann, M. and Landgrat, R. (1998) The effects of infusion of arginine vasopressin, oxytocin, or their antagonists into the olfactory bulb upon social recognition responses in male rats. *Peptides vol. 19, No. 6, P999-1005*

Domes, G., Heinrichs, M., Michel, A., Berger, C. and Herpertz, S.C. (2007) Oxytocin improves "mind-reading" in humans. *Biological Psychiatry, vol. 61, no. 6, P731-733*

Dong N., Dong J., Liu P., Xu L., Shi S. and Wu Q. (2010) Effects of anticoagulants on human plasma soluble corin levels measured by ELISA. *Clinica Chimica Acta 414, No. 23, P1998-2003.*

Drewett, R.F., Bowen-Jones, A. and Dogterom, J. (1982) Oxytocin levels during breast-feeding in established lactation. *Hormones and Behaviour vol 16, No. 2, P245-248*

Dudkiewicz-Wilczynska, J., Snycerski, A. And Tautt, J. (2000) HPLC method for the determination of oxytocin in pharmaceutical dosage form and comparison with biological method. *Acta poloniae pharmaceutica vol 57, no 6, P403-406*

Dwyer.C.M. (2008) Individual variation in the expression of maternal behaviour: a review of the neuroendocrine mechanisms in the sheep. *Journal of Neuroendocrinology vol 20, P526-534*

El-Masry, N., Soliman, A. and Moety, H.A. (2010) Alterations of prolyl endopeptidase, oxytocin and vasopressin activity in the plasma of autistic children. *Current Psychiatry* vol 17, No. 1, P31-37

Ermisch, A., Rühle, H.J., Landgraf, R. and Hess, J. (1985a) Blood-brain barrier and peptides. *Journal of Cerebral Blood Flow and Metabolism vol 5, P350-357*

Ermisch, A, Barth, T., Rühle, H.J., Skopková, J., Hrbas, P. and Landgraf, R. (1985b) On the blood-brain barrier to peptides: accumulation of labelled vasopressin, DesGlyNH2-vasopressin and oxytocin by brain regions. *Endocrinologia experimentalis vol 19, No. 1, P29*

Estevez, I., Keeling, L.J. and Newberry, R.C. (2003) Decreasing aggression with increasing group size in young domestic fowl. *Applied Animal Behaviour Science vol 84, No. 3, P213-218*

Extavour, C.G.M. (2007) Evolution of the bilaterian germ line: lineage origin and modulation of specification mechanisms. *Intergrative and Comparative Biology vol 47, No 5, P770-785*

Fairbanks, L.A. (1996) Individual differences in maternal style: causes and consequences for mothers and offspring. *Advances in the Study of Behaviour vol 25, P579-611*

Fedak M.A. and Anderson S.S. (1982) The energetics of lactation: accurate measurements from a large wild mammal, the grey seal (*Halichoerus grypus*). *Journal of Zoology vol 198, P473-479*

Feldman, R., Weller, A., Zagoory-Sharon, O. and Levine A. (2007) Evidence for a neuroendocrinological foundation of human affiliation: plasma oxytocin levels across pregnancy and the postpartum period predict mother-infant bonding. *Psychological Science vol 18, No.11, P965-970*

Feldman, R., Gordon, I., Schneiderman, I., Weisman, O. and Zagoory-Sharon, O. (2010a) Natural variations in maternal and paternal care are associated with systematic changes in oxytocin following parent-infant contact. *Psychoneuroendocrinology vol 35, No. 8, P113-1141*

Feldman, R., Gordon, I. and Zagoory-Sharon, O. (2010b) The cross-generation transmission of oxytocin in humans. *Hormones and Behaviour vol 58, No. 4, P669-676*

Ferguson, J.N., Young, L.J., Hearn, E.F., Matzuk, M.M., Insel, T.R. and Winslow, J.T. (2000) Social amnesia in mice lacking the oxytocin gene. *Nature Genetics Vol 25, No. 3, P284-288*

Ferguson, J.N., Aldag, J.M., Insel, T.R. and Young, L.J. (2001) Oxytocin in the medial amygdala is essential for social recognition in the mouse. *Journal of Neuroscience vol 21, No. 20, P8278-8285*

Ferguson, J.N., Young, L.J. and Insel, T.R. (2002) The neuroendocrine basis of social recognition. *Frontiers in Neuroendocrinology vol 23, No. 2, P200-224*

Ferrier, B.M., Hendrie, J.M. and Branda , L.A. (1974) Plasma oxytocinase: the synthesis and biological properties of the first product of the degradation of oxytocin be this enzyme. *Canadian Journal of Biochemistry vol 52, No. 1, P60-66*

Fisher, J. (1954) Evolution and bird sociality. *In Huxley, J., Hardy, A.C. and Ford. E.B. ed. Evolution as a Process. Allen and Unwin, London, UK P71-83*

Fjellestad-Paulsen, A., Söderberg-Ahlm, C. and Lundin, S. (1995) Metabolism of vasopressin, oxytocin and their analogues in the human gastrointestinal tract. *Peptides vol 16, No. 6, P1141-1147*

Fogden, S.C.L. (1971) Mother-young behaviour at grey seal breeding beaches. *Journal of Zoology vol 164, No. 1, P61-92*

Francis, D.D., Champagne, F.C. and Meaney, M.J. (2000) Variations in maternal behaviour are associated with difference in oxytocin receptor levels in the rat. *Journal of Neuroendocrinology vol 12, No. 12, P1145-1148*

Francis, D.D., Young, L.J., Meaney, M.J. and Insel, T.R. (2002) Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. *Journal of Neuroendocrinology vol 14, No. 5, P349-353*

Gimpl, G. and Fahrenholz, F. (2001) The oxytocin receptor system, structure function and regulation. *Physiology Review vol 81, No. 2, P629-683*

Gingrich, B., Liu, Y., Casico, C., Wang, Z. and Insel, T.R. (2000) Dopamine D2 receptors in the nucleus accumbens are important for social attachment in female prairie voles (*Microtus ochrogaster*). *Behavioural Neuroscience vol 114, No. 1, P173-183*

Ginsburg, M., Smith, M.W. (1959) The fate of oxytocin in male and female rats. *British Journal of Pharmacology and Chemotherapy vol 14, No. 3, P327-333*

Gonzalez-Covarrubias V., Dane A., Hankemeier T. and Vreeken RJ. (2013) The influence of citrate, EDTA and heparin anticoagulants to human plasma LC-MS lipidomic profiling. *Metabolomics vol 9, P337-348*

Goodson, J.L., Schrock, S.E., Klatt, J.D., Kabelik, D. and Kingsbury, M.A. (2009) Mesotocin and nonapeptide receptors promote estrildid flocking behavior. *Science vol 325, No. 5942, P862-866*

Gordon, I., Zagoory-Sharon, O., Leckman, J.F. and Feldman, R. (2010) Oxytocin, cortisol and triadic family interactions. *Physiology and Behaviour vol 101, No. 5, P679-684*

Gosling, S.D. and John, O.P. (1999) Personality dimensions in nonhuman animals: a cross species review. *Current Directions in Psychologcial Science vol 8, No. 3, P69-75*

Gossen, A., Hahn, A., Westphal, L., Prinz, S., Schultz, R.T., Gründer, G. and Speckelmeyer, K.N. (2012) Oxytocin plasma concentrations after single intranasal oxytocin administration – a study in healthy men." *Neuropeptides vol 46, No. 5, P211-215*

Gould, S.J. and Lewontin R.C. (1979) The spandrels of San Marco and the Panglossian Paradigm: a critique of the adaptationist programme. *Proceedings of the Royal Society B vol 205, No. 1161, P581-598*

Goymann, W. and Hofer, H. (2010) Mating systems, social behaviour and hormones. *In Kappeler, P. ed. Animal Behaviour: Evolution and Mechanisms. Springer-Verlag, Berlin, Germany P465-501*

Grewen, K.M., Girdler, S.S., Amico, J. and Light, K.C.L (2005) Effect of partner support on resting oxytocin, norepinephrine and blood pressure before and after warm partner contact. *Psychosomatic Medicine vol 67, No. 4, P531-538*

Grewen, K.M., Davenport, R.E. and Light, K.C. (2010) An investigation of plasma and salivary oxytocin responses in breast and formula feeding mothers of infants. *Psychophysiology, vol 47, No. 4, P625-632.*

Grippo, A.J., Trahanas, D.M., Zimmerman, R.R., 2nd, Porges, S.W. and Carter, C.S. (2009) Oxytocin protects against negative behavioural and autonomic consequences of long-term social isolation. *Psychoneuroendocrinology vol 34, No. 10, P1542-1553*

Guastella, A.J., Mitchell, P.B. and Mathews, F. (2008) Oxytocin enhances the encoding of positive social memories in humans. *Biological Psychiatry vol 64, No. 3, P256-258*

Haaland P., Samarov D. and McVey E. (2011) calibFit: Statistical models and tools for assay calibration. R package version 2.1.0/r17. http://R-Forge.R-project.org/projects/calibfun/

Hall, A.J. (1998) Blood chemistry and haematology of gray seal (*Halichoerus grypus*) pups from birth to postweaning. *Journal of Zoo and Wildlife Medicine vol 29, No. 4, P401-407*

Hall, A.J., McConnell, B.J. and Barker, R.J. (2001) Factors affecting first-year survival in grey seals and their implications for life history strategy. *Journal of Animal Ecology vol. 70, No. 1, P138-149*

Hall, A.J., McConnell, B.J. and Barker, R.J. (2002) The effect of total immunoglobulin levels, mass and condition on the first year survival of grey seal pups. *Functional Ecology vol 16, P462-474*

Haller, M.A., Kovacs, K.M. and Hammill, M.O. (1996) Maternal behaviour and energy investment by grey seals (*Halichoerus grypus)* breeding on land-fast ice. *Canadian Journal of Zoology vol 74, P1531-1541*

Hammill, M.O and Gosselin, J.F. (1995) Grey seal (*Halichoerus grypus*) from the Northwest Atlantic: female reproductive rates, age at first birth, and age of maturity in males. *Canadian Journal of Fisheries and Aquatic Sciences vol 52, No. 12, P2757-2761*

Hammock, E.A.D and Young, L.J. (2006) Oxytocin, vasopressin and pair bonding: implications for autism. *Philosophical Transactions of the Royal Society B: Biological Sciences vol 361, No. 1476, P2187-2198*

Harcourt, R.G., Turner, A., Hall, A., Waas, J.R. and Hindell, M. (2010) Effects of capture stress on free-ranging, reproductively active male Weddell seals. *Journal of Comparative Physiology A vol 196, No. 2, P147-154*

Hardy, A., Clevenger, A.P., Marcel, H. and Graham, N. (2003) An overview of methods and approaches for evaluating the effectiveness of wildlife crossing structures: emphasizing the science in applied science. *The Road Ecology Center, University of California*

Heinrichs, M., Baumgartner, T. Kirschbaum, C. and Ehlert, U. (2003) Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. *Biological Psychiatry vol 54, No. 12, P1389-1398*

Heinrichs, M. and Domes, G. (2008) Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. *Progress in Brain Research vol. 170, P337-350*

Herrero, S. (1983) Social behaviour of black bears at a garbage dump in Jasper national park. *Bears: Their Biology and Management vol 5, P54-70*

Hewer, H.R. (1974) British Seals. *Taplinger Pub. Co. New York, USA.*

Higuchi, T., Honda, K., Fukuoka, T., Negoro, H., and Wakabayashi, K. (1985) Release of oxytocin during suckling and parturition in the rat. *Journal of Endocrinology vol 105, P339-346*

Hoffman E.R., Brownley K.A., Hamer R.M. and Bulik C.M. (2012) Plasma, salivary and urinary oxytocin in anorexia nervosa: a pilot study. *Eating Behaviours vol 13, No. 3, P256-259*

Hoge, E.A., Lawson, E.A., Metcalf, C.A., Keshaviah, A., Zak, P.J., Pollack, M.H. and Simon, N.M. (2012) Plasma oxytocin immunoreactive products and response to trust in patients with social anxiety disorder. *Depression and Anxiety vol 29, No. 11, P924-930*

Hollander, E., Bartz, J., Chaplin, W., Phillips, A., Sumner, J., Soorya, L., Anagnostou, E. and Wasserman, S. (2007) Oxytocin increases retention of social cognition in autism. *Biological Psychiatry vol 61. No. 4, P498-503*

Holt-Lundstad, J., Birmingham, W. and Light, K.C. (2011) The influence of depressive symptomatology and perceived stress on plasma and salivary oxytocin before, during and after a support enhancement intervention. *Psychoneuroendocrinology vol 36, No. 8, P1249-1256*

Homeida, A.M. and Cooke, R.G. (1984) Biological half-life of oxytocin in the goat. *Research in Veterinary Science vol 37, No. 3, P364-365*

Horvat-Gordon, M., Granger, D.A., Schwartz, E.B., Nelson, V.J. and Kivlighan, K.T. (2005) Oxytocin is not a valid biomarker when measured in saliva by immunoassay. *Physiology and Behaviour vol 84, No. 8, P445-448*

Hrdy, S.B. (1979) Infanticide among animals: a review, classification and examination of the implications for the reproductive strategies of females. *Ethology and Sociobiology vol 1, No. 1, P13-40*

Hurlemann, R., Patin A, Onur, O.A., Cohen, M.X., Baumgartner T., Metzler, S., Dziobek, I., Gallinat, J., Wagner, M., Maier, W. and Kendrick, K.M. (2010) Oxytocin enchances amygdala-dependant, socially reinforced learning and emotional empathy in humans. *The Journal of Neuroscience vol 30, No. 14, P4999-5007*

Insel, T. R. and Shapiro, L. E. (1992) Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles. *Proceedins of the National Academy of Science USA vol 89, No. 13, P5981-5985*

Insley, S. J., Phillips, A.V. and Charrier, I. (2003) A review of social recognition in pinnipeds. *Aquatic Mammals, vol. 29, P181-201*

Iverson, S.J., Bowen, W.D., Boness, D.J. and Oftedal, O.T. (1993) The effect of maternal size and milk energy output on pup growth in grey seals (*Halichoerus grypus*). *Physiological Zoology vol 66, P61-88*

Iwata, M. (1995) Downstream migratory behaviour of salmonids and its relationship with cortisol and thyroid hormones: a review. *Aquaculture vol 135, No. 1-3, P131-139*

Johnson, J.C. and Sih, A. (2005) Precopulatory sexual cannibalism in fishing spiders (*Dolomedes triton*): a role for behavioural syndromes. *Behaivoural Ecology and Sociobiology vol 58, No. 4, P390-396*

Kagerbauer, S.M, Martin, J., Schuster, T., Blobner, M., Kochs, E.F. and Landgraf, R. (2013) Plasma oxytocin and vasopressin do not predict neuropeptide concentrations in human cerebrospinal fluid. *Journal of Neuroendocrinology vol 25, No. 7, P668-673*

Kalin, N.H., Gibbs, D.M., Barksdale, C.M., Shelton, S.E. and Carnes, M. (1985) Behavioural stress decreases plasma oxytocin concentrations in primates. *Life Sciences vol 26, No. 13, P1275-1280*

Kang, Y.S. and Park, J.H. (2000) Brain uptake and the analgesic effect of oxytocin – its usefulness as an analgesic agent. *Archives of Pharmacal Research vol 23, No. 4, P391-395*

Kendrick, K.M., Keverne, E.B., Hinton M.R. and Goode J.A. (1991a) Cerebrospinal fluid and plasma concentrations of oxytocin and vasopressin during parturition and vaginocervical stimulation in the sheep. *Brain Research Bulletin Vol 26, No. 5, P 803-807*

Kendrick, K.M. and Keverne, E.B. (1991b) Importance of vaginocervical stimulation for the formation of maternal bonding in primiparous and multiparous parturient ewes. *Physiology & Behavior Vol 50, No. 3,, Pages 595-600*

Kendrick, K.M., Keverne, E.B., Hinton, M.R. and Goode, J.A. (1992) Oxytocin, amino acid and monoamine release in the regions of the medial preoptic area and bed nucleus of the stria terminalis of the sheep during parturition and suckling. *Brain Research vol 569, No. 2, P199-209*

Kendrick, K.M., Da Costa, A.P.C., Broad, K.D., Ohkura, S., Guevara, R., Lévy, F. And Keverne E.B. (1997) Neural control of maternal behaviour and olfactory recognition of offspring. *Brain Research Bulletin vol 44, No. 4, P383-395*

Kendrick, K.M. (2000) Oxytocin, motherhood and bonding. *Experimental Physiology, vol 85, no. 1, P111-124*

Kleiman, D.G. and Eisenberg, J.F. (1973) Comparisons of canid and felid social systems from an evolutionary perspective. *Animal Behaviour vol 21, No. 4, P637-659*

Kondo, N., Sekijima, T., Kondo, J., Takamatsu, N., Tohya, K. and Ohtsu, T. (2006) Circannual control of hibernation by HP complex in the brain. *Cell vol 125, No. 1, P161-172*

Kooymann, G.L. (1989) Diverse Divers, Physiology and Behaviour. *Springer-Verlag, Berlin, Germany*

Kosfeld, M., Heinrichs, M., Zak, P.J. Fischbacher, U. and Fehr, E. (2005) Oxytocin increases trust in humans. *Nature vol 435, P673-676*

Kovacs, K.M. (1987) Maternal behaviour and early behavioural ontogeny of grey seals (*Halichoerus grypus*) on the Isle of May, UK. *Journal of Zoology vol 213, No. 4, P697-715*

Kovacs, K.M. and Innes, S. (1990) The impact of tourism on harp seals (*Phoca groenlandica*) in the Gulf of St Lawrence, Canada. *Applied Animal Behaviour Science vol 26, No. 1-2, P15-26*

Kowalski, W.B., Diveky, L., Mehendale, R., Parsons, M. And Wilson Jr, L. (1998) Effect of pregnancy on the metabolic clearance rate and the volume of distribution of oxytocin in the baboon. *American Journal of Physiology Endocrinology and Metabolism vol 274, P791-795*

Kramer, K.M., Cushing, B.S., Carter, C.S., Wu, J. and Ottinger, M.A. (2004) Se and species differences in plasma oxytocin using an enzyme immunoassay. *Canadian Journal of Zoology vol 84, No. 8, P1194-1200*

Lair, S., Michaud, R., Measures, L., Béland, P. and Ménard, N (2013) Marked increase in perinatal mortalities in beluga (*Delphinapterus leucas*) from the St Lawrence Estuary, Quebec, Canada. *Talk presentation at: 20th Biennial Conference on the Biology of Marine Mammals, 2013 9-13 December, Dunedin, New Zealand*

Landgraf, R. (1985) Plasma oxytocin concentrations in man after different routes of administration of synthetic oxytocin. *Experimental and Clinical Endocrinology and Diabetes vol 85, No. 2, P245-248*

Landgraf, R. and Neumann, I.D. (2004) Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Frontiers in Neuroendocrinology vol 25, No. 3-4, P150-176*

Langeland, A. and Dijkstra, S. (1995) Breaking the intergenerational transmission of child abuse: beyond the mother-child relationship. *Child Abuse Review vol 4, No. 1, P4-13*

Leake, R.D., Weitzman, R.E. and Fisher, D.A. (1980) Pharmokinetics of oxytocin in the human subject. *Obstetrices and Gynecology vol 56, No. 6, P701-704*

Leake, R.D., Weitzman, R.E. and Fisher, D.A. (1981) Oxytocin concentrations during the neonatal period. *Biol Neonate vol. 39, No. 3-4, P127-31*

Legros, J.J., Chiodera, P., Geenen, V., Smitz, S. and von Frenckell, R. (1984) Dose-response relationship between plasma oxytocin and cortisol adrenocorticotropin concentrations during oxytocin infusions in normal men. *The Journal of Clinical Endocrinology and Metabolism vol 58, No. 1, P105-109*

Legros, J.J., Chiodera, P., Geenen, V. and von Frenckell (1987) Confirmation of the inhibitory influence of exogenous oxytocin on cortisol and ACTH in man: evidence of reproducibility. *Acta Endocrinologica vol 114, No. 3 P345-349*

Leiser, J.K. (2003) When are neighbours 'dear enemies' and when are they not? The responses of territorial male variegated pupfish, *Cyprinodon variegates,* to neighbours, strangers and heterospecifics. *Animal Behaviour vol 65, No. 3, P453-462*

Levine, A., Zagoory-Sharon, O., Feldman, R. and Weller, A. (2007) Oxytocin during pregnancy and early postpartum: Individual patterns and maternal-fetal attachment. *Peptides vol 28, No. 6, P1162-1169*

Lévy, F., Kendrick, K.M., Keverne, E.B., Piketty, V. and Poindron, P. (1992) Intracerebral oxytocin is important for the onset of maternal behaviour in inexperienced ewes delivered under peridural anesthesia. *Behavioural Neuroscience vol 106, No. 2, P427-432*

Lévy, F., Kendrick, K. M., Goode, J. A., Guevara-Guzman, R. and Keverne, E. B. (1995) Oxytocin and vasopressin release in the olfactory bulb of parturient ewes: changes with maternal experience and effects on acetylcholine, gamma-

aminobutyric acid, glutamate and noradrenaline release. *Brain Research vol 669, No. 2, P197-206*

Li, L.L., Keverne, E.B., Aparicio, S.A., Ishino, F., Barton, S.C. and Surani, M.A. (1999) Regulation of maternal behavior and offspring growth by paternally expressed Peg3. *Science vol 284, No. 5412, P330-334*

Lidfors, L.M., Jensen, P. and Algers, B. (1994) Suckling in free-ranging beef cattle – temporal patterning of suckling bouts and effects of age and sex. *Ethology vol 98, No. 3-4, P321-332*

Lidgard, D.C., Bowen, W.D., Jonsen, I.D. and Iverson, S.J. (2012) Animal-borne acoustic transceivers reveal patterns of at-sea associations in an upper trophic level predator. *PLOS one vol 7, No. 11, e48962*

Lim, M.M. and Young, L.J. (2006) Neuropeptidergic regulation of affiliative behavior and social bonding in animals. *Hormones and behaviour vol 50, No. 4, P506-517*

Lipari, E.F., Lipari, D, Gerbino, A., Di Liberto, D., Bellafiore, M., Catalano, M. And Valentino, B. (2009) The hypothalamic magnocellular neurosecretory system in developing rats. *European Journal of Histochemistry vol 45, No. 2, P163-168*

Lundin, S., Broeders, A., Ohlin, M., Hansson, K., Bengtsson, H.I., Trojnar, J. and Melin, P. (1993) Pharmacokinetic and pharmacologic properties of antiuterotonic oxytocin analogs in the rat. *The Journal of Pharmacology vol 264, No. 2, P783-788*

Lydersen, C., Hammill, M.O., and Kovacs, K.M. (1995) Milk intake, growth and energy consumption in pups of ice-breeding grey seals (*Halichoerus grypus*) from the Gulf of St Lawrence, Canada. *Journal of Comparative Physiology B vol 164, P585-592*

MacDonald, K. and MacDonald, T.M. (2010) The peptide that binds: a systematic review of oxytocin and its prosocial effects in humans. *Harvard Review of Psychiatry vol 18, No. 1, P1-21*

Madden, J.R. and Clutton-Brock, T.H. (2011) Experimental peripheral administration of oxytocin elevates a suite of cooperative behaviours in a wild social mammal. *Proceedings of the Royal Society B vol 278, No. 1709, P1189-1194*

Maestripieri D., Hoffman C.L., Anderson G.M, Carter C.S., Higley J.D. (2009) Mother-infant interactions in free-ranging rhesus macaques: relationships between physiological and behavioural variables. *Physiology and Behaviour vol 96, No. 4, P 613-619*

Main, M. and Goldwyn, R. (1984) Predicting rejection of her infant from mother's representation of her own experience: implications for the abused-abusing intergenerational cycle. *Child Abuse and Neglect vol 8, No. 2, P203-217*

Marazziti, D., Dell'Osso, B., Baroni, S., Mungai, F., Catena, M., Rucci, P., Albanese, F., Giannaccini, G., Betti, L., Fabbrini, L., Italiani, P., Del Debbio, A., Lucacchini, A. And Dell'Osso, L. (2006) A relationship between oxytocin and anxiety of romatic attachment. *Clinical Practice and Epidemiology in Mental Health vol 2, No. 1, P28*

Martínez-Lorenzana, G., Espinosa-López, L., Carranza, M., Aramburo, C., Paz-Tres, C., Rojas-Piloni, G. and Condés-Lara, M. (2008) PVN electrical stimulation prolongs withdrawal latencies and releases oxytocin in cerebrospinal fluid, plasma, and spinal cord tissue in intact and neuropathic rats. *Pain vol 140, No. 2, P265 – 273*

Maynard Smith, J. (1977) Parental investment: a prospective analysis. *Animal Behaviour vol 25, P1-9*

McCann, T.S. (1980) Territoriality and breeding behaviour of adult male Antarctic fur seal, *Arctocephalus gazelle*. *Journal of Zoology, vol 192, No. 3, P295-310*

McCarthy, M.M. (1990) Oxytocin inhibits infanticide in female house mice (*Mus domesticus*) *Hormones and Behavior Volume 24, No. 3, Pages 365-375*

McCulloch, S., Pomeroy, P.P. and Slater, P.J.B. (1999) Individually distinctive pup vocalizations fail to prevent allo-suckling in grey seals. *Canadian Journal of Zoology vol. 77, P716-723*

McCulloch, S. and Boness, D.J. (2000) Mother-pup vocal recognition in the grey seal (*Halichoerus grypus*) of Sable Island, Nova Scotia, Canada. *Journal of Zoology vol. 251, No.4, P449-455*

McCullough, M.E., Churchland, P.S. and Mendez, A.J. (2013) Problems with measuring peripheral oxytocin: can the data on oxytocin and human behaviour be trusted? *Neuroscience and Biobehavioural Reviews vol 37, No. 8, P1485-1492*

McNamara, J.M and Houston, A.I. (2008) Optimal annual routines: behaviour in the contect of physiology and ecology. *Philosophical Transactions of the Royal Society B vol 363, No. 1490, P301-319*

McNeilly, A.S., Robinson, I.C., Houston, M.J. and Howie, P.W. (1983) Release of oxytocin and prolactin in response to suckling. *British Medical Journal vol 286, P257-259*

Mellish, J.E., Iverson, S.J. and Bowen, W.D. (1999) Variation in milk production and lactation performance in grey seals and consequences for pup growth and weaning characteristics. *Physiological and Biochemical Zoology vol 76. No. 6, P677-690*

Mens, W.B.J., Witter, A. and Van Wimersma Greidanus, T.B. (1983) Penetration of neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): half-times of disappearance of the neuropeptides from CSF. *Brain Research vol 262, No. 1, P143-149*

Michopoulos, V., Checchi, M., Sharpe, D. and Wilson, M.E. (2011) Estradiol effects on behaviour and serum oxytocin are modified by social status and polymorphisms in the serotonin transporter gene in female rhesus monkeys. *Hormones and Behaviour vol 59, No. 4, P528-535*

Miller, E.H. and Boness, D.J. (1983) Summer behaviour of the Atlantic walrus (*Odobenus rosmarus*) on Coats Island, NWT. *Zeitschrift für Säugetierkunde vol 48, P298-313*

Modahl, C., Green, L.A., Fein, D., Morris, M., Waterhouse, L., Feinstein, C. and Levin, H. (1998) Plasma oxytocin levels in autistic children. *Biological Psychiatry vol 43, No. 4, P270-277*

Moos, F., Freund-Mercier, M.J., Guerné, Y., Guerné, J.M., Stoeckel, M.E. and Richard, P. (1984) Release of oxytocin and vasopressin by magnocellular nuclei *in vitro*: specific facilitatory effect of oxytocin on its own release. *Journal of Endocrinology vol 102, No. 1, P63-NP*

Morrin, V., del Castillo, J.R.E., Authier, S., Ybarra, N., Otis, C., Gauvin, D., Gutkowska, J. and Troncy, E. (2008) Evidence for non-linear phatmacokinetics of oxytocin in anesthetized rat. *Journal of pharmacy & pharmaceutical sciences: a publication of the Canadian Society for Pharmaceutical Sciences, Société canadienne des sciences pharmaceutiques vol 11, No. 4, P12-24*

Moscovice, L.R. and Ziegler, T.E. (2012) Peripheral oxytocin in female Chacma baboons relates to estrus and maintenance of sexual consortships. *Hormones and Behaviour vol 62, No. 5, P592-597*

Nagasawa, M., Kikusui, T., Onaka, T. and Ohta, M. (2009) Dog's gaze at its owner increases owner's urinary oxytocin during social interaction. *Hormones and Behaviour vol 55, No. 3, P434-441*

Nagasawa, M., Okabe, S., Mogi, K. and Kikusui, T. (2012) Oxytocin and mutual communication in mother-infant bonding. *Frontiers in Human Neuroscience vol 6, P31*

Nelson, E. and Panksepp, J. (1996) Oxytocin mediates acquisition of maternally associated odor preferences in preweanling rat pups. *Behavioural Neuroscience vol 110, No. 3, P583-592*

Nelson, R.J. (2000) An Introduction to Behavioural Endocrinology. *2nd Edition, Sinauer Associates Inc, Massachusetts, USA.*

Neumann, I., Douglas, A.J., Pittman, Q.J., Russell, J.A. and Landgraf, R. (1996) Oxytocin released within the supraoptic nucleus of the rat brain by positive feedback action is involved in parturition-related events. *Journal of Neuroendocrinology vol 8. No. 3, P227-233*

Odendaal, J.S.J. and Meintjes, R.A. (2003) Neurophysiological correlates of affiliative behaviour between humans and dogs. *The Veterinary Journal vol 165, P296-301*

Olausson, H., Uvnäs-Moberg, K. and Sohlström, A. (2003) Postnatal oxytocin alleviates adverse effects in adult rat offspring caused by maternal malnutrition. *American Journal of Physiology – Endocrinology and Metabolism vol 284, No. 3, P475-480*

Olazábal, D.E. and Young, L.J. (2006) Oxytocin receptors in the nucleus accumbens facilitate “spontaneous” maternal behaviour in adult female prairie voles. *Neuroscience vol 141, No. 2, P559-568*

Oldfield, R.G. and Hofmann, H.A. (2011) Neuropeptide regulation of social behaviour in a monogamous cichlid fish. *Physiology and Behaviour vol 102, No.3-4, P296-303*

Pendersen, A. and Tomaszycki, M.L. (2012) Oxytocin antagonist treatments alter the formation of pair relationships in zebra finches of both sexes. *Hormones and Behaviour vol 62, No. 2, P113-119*

Perry, E.A., Boness, D.J. and Fleischer, R.C. (1998) DNA Fingerprinting evidence of nonfilial nursing in grey seals. *Molecular Ecology vol. 7, No. 1, P81-85*

Petrovic, P., Kalisch, R., Singer, T. and Dolan, R.J. (2008) Oxytocin attenuates affective evaluations of conditioned faces and amygdala activity. *Journal of Neuroscience, vol 28, no. 26, P6607-6615*

Phaneuf, S., Linares, B.R. TambyRaja, R.L., MacKenzie, I.Z. and Bernal, A.L. (2000) Loss of myometrial oxytocin receptors during oxytocin induced and oxytocin-augmented labour. *Journal of Reproduction and Fertility vol 120, No. 1, P91-97*

Pitcher, B.J., Harcourt, R.G., Schaal, B. and Charrier, I. (2011) Social olfaction in marine mammals: wild female Australian sea lions can identify their pup's scent. *Biology Letters vol 7, No. 1, P60-62*

Poland, V.F., Pomeroy, P.P., Twiss, S.D. and Graves J.A. (2008) Fine-scale study finds limited evidence of kin clustering in a gray seal colony. *Marine Mammal Science vol 24, No.2, P371-387*

Popik, P., Vetulani, J. and van Ree, J.M. (1992) Low doses of oxytocin facilitate social recognition in rats. *Psychopharmacology vol 106, P71-74*

Pomeroy, P.P., Anderson, S.S., Twiss, S.D. and McConnell (1994) Dispersion and site fidelity of breeding female grey seals (*Halichoerus grypus*) on North Rona, Scotland. *Journal of Zoology vol 233, No. 3, P429-447*

Pomeroy, P.P., Fedak, M.A., Rothery, P. and Anderson, S. (1999) Consequences of maternal size for reproductive expenditure and pupping success of grey seals at North Rona, Scotland. *Journal of Animal Ecology vol. 68, No. 2, P235-253*

Pomeroy, P.P., Twiss, S.D. and Redman, P. (2000) Philopatry, site fidelity and local kin associations within grey seal breeding colonies. *Ethology vol.106, No. 10. P899-919*

Pomeroy, P.P., Worthington Wilmer, J., Amos, W. and Twiss, S.D. (2001) Reproductive performance links to fine-scale spatial patterns of female grey seal relatedness. *Proceedings of The Royal Society of London B vol 268, No. 1468, P711-717*

Pomeroy, P.P., Redman, P.R., Ruddell, S.J.S., Duck, C.D. and Twiss, S.D. (2005) Breeding site choice fails to explain interannual associations of female grey seals. *Behavioral Ecology and Sociobiology vol 57, No. 6, P546-556*

Prakash, B.S., Paul, V., Kliem, H., Kulozik, U. and Meyer, H.H.D. (2009) Determination of oxytocin in milk of cows administered oxytocin. *Analytica Chimica Acta vol 636, No. 1, P111-115*

Ragnauth, A.K., Devidze, N., Moy, V., Finley, K., Goodwillie, A., Kow, L.M., Muglia L.J. and Pfaff, D.W. (2005) Female oxytocin gene-knockout mice, in a semi-natural environment, display exaggerated aggressive behavior. *Genes, Brain & Behavior, vol 4, no. 4, P229-239*

Ratti, J.T. and Garton, E.O. (1994) Research and experimental design. *Research and Management Techniques for Wildlife and Habitats, fifth edition. The Wildlife Society, Bethesda, Maryland, USA P1-23*

Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E. and Balshine, S. (2012) Effects of isotocin on social responses in a cooperatively breeding fish. *Animal Behaviour vol 84, No. 4, P753-760*

Redman, P., Pomeroy, P.P., and Twiss, S.D. (2001) Grey seal maternal attendance patterns are affected by water availability on North Rona, Scotland. *Canadian Journal of Zoology vol 79, No. 6, P1073-1079*

Redman, P. (2002) The role of temporal, spatial and kin associations in grey seal breeding colonies. *PhD thesis, The University of St Andrews, Scotland*

Reilly, J.J. (1991) Adaptions to prolonged fasting in free-living weaned gray seal pups. *American Journal of Physiology – Regulatory, Intergrative and Comparative Physiology vol 260, No. 2, P267-272*

Reinhardt, V. and Reinhardt, A. (1981) Natural suckling performance and age of weaning in zebu cattle (*Bos indicus*). *The Journal of Agriculture Science vol 95, No. 2, P309-312*

Renouf, D and Diemand, D. (1984) Behavioural interactions between harbour seal mothers and pups during weaning (Pinnipeds: Phocidae). *Mammalia vol 48, No. 1, P53-58*

Revelle, W. (2013) psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA, http://CRAN.R-project.org/package=psych

Richards, S.A., Whittingham, M.J. and Stephens, P.A. (2011) Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. *Behavioural Ecology and Sociobiology vol 65, No. 1, P77-89*

Rimmele, U., Hediger, K., Heinrichs, M. and Klaver, P. (2009) Oxytocin makes a face in memory familiar. *Journal of Neuroscience vol 29, No. 1, P38-42*

Robinson, I.C.A.F. (1980) The development and evaluation of a sensitive and specific radioimmunoassay for oxytocin in unextracted plasma. *Journal of Immunoassay vol 1, No. 3, P323-347*

Robinson, C., Schumann, R., Zhang, P. and Young, R.C. (2003) Oxytocin-induced desensitization of the oxytocin receptor. *American Journal of Obstetrics and Gynecology vol 188, No. 2, P497-502*

Roger, K., Smith, A., Davis, C., Newsome, D. and Patterson, P. (2010) A framework to guide the sustainability of wildlife tourism operations: examples of marine wildlife tourism in Western Australia. *CRC for Sustainable Tourism*

Romesburg, H.C. (1981) Wildlife science: gaining reliable knowledge. *The Journal of Wildlife Management vol 45, No. 2, P293-313*

Rosell, F. and Bjørkøyli, T. (2002) A test of the dear enemy phenomenon in the Eurasian beaver. *Animal Behaviour vol 63, No. 6, P1073-2078*

Ross, H.E., Freeman, S.M., Spiegel, L.L., Ren, X., Terwilliger, E.F. and Young, L.J. (2009) Variation in oxytocin receptor density in the nucleus accumbens has differential effects on affiliative behaviors in monogamous and polygamous voles. *Journal of Neuroscience vol. 29, No. 5, P1312-1318*

Rougier, N. and Webb, Z. (2001) Minireview: Parthenogenesis in Mammals. *Molecular Reproduction and Development vol 59, No. 4, P468-474*

Ruddell, S. (2005) Sociality of female grey seals breeding on North Rona, Scotland. *PhD thesis, the University of St Andrews, Scotland*

Ruddell, S.J.S., Twiss, S.D. and Pomeroy, P.P. (2007) Measuring opportunity for sociality: quantifying social stability in a colonially breeding phocid. *Animal Behaviour vol. 74, No.5, P1357-1368*

Savaskan, E., Ehrhardt, R., Schulz, A., Walter, M. and Schächinger, H. (2008) Post-learning intranasal oxytocin modulates human memory for facial identity. *Psychoneuroendocrinology vol 33, No. 3, P368-374*

Scantamburlo G., Hansenne M., Fuchs S., Pitchot W., Marechal P., Pequeux C., Ansseau M. and Legros J.J. (2007) Plasma oxytocin levels and anxiety in patients with major depression. *Psychoneuroendocrinology vol 32, No. 4, P407-410*

Schmidt, A.J., Bmvenuti, C.E. and Diele, K. (2012) Effects of geophysical cycles on the rythmes of mass mate searching of a harvested mangrove crab. *Animal Behaviour vol 84, No. 2, P333-340*

Shapiro, L.E. and Insel, T.R. (1989) Ontogeny of oxytocin receptors in rat forebrain: a quantitative study. *Synapse vol 4, No. 3, P259-266*

Sheldrick, E.L. and Flint, A.P.F. (1981) Circulation concentrations of oxytocin during the estrous cycle and early pregnancy in sheep. *Prostaglandins vol 22, No. 4, P631-636*

Shine, R. (2003) Reproductive strategies in snakes. *Proceedings of the Royal Society B vol 270, N0. 1519, P995-1004*

Sih, A., Bell, A. and Johnson, J.C. (2004) Behavioural syndromes: an ecological and evolutionary overview. *Trends in Ecology and Evolution vol 19, No. 7, P372-378*

Skrundz M., Bolten M., Nast I., Hellhammer D.H. and Meinlschmidt G. (2011) Plasma oxytocin concentration during pregnancy is associated with development of postpartum depression. *Neuropsychopharmacology vol 36, No. 9, P1886-1893*

Smith, A.S., Agmo, A., Birnie, A.K. and French, J.A. (2010) Manipulation of the oxytocin system alters social behaviour and attraction in pair-bonding primates, *Callithrix penicillata*. *Hormones and Behaviour vol 57, No. 2, P255-262*

Sohlström, A., Carlsson, C. and Uvnäs-Moberg, K. (2000) Effects of oxytocin treatment in early life on body weight and corticosterone in adult offspring from ad libitum-fed and food restricted rats. *Biol Neonate vol 73, P33-40*

Spotte. S. and Stake P.E. (1982) Hand rearing of twin gray seals (*Halichoerus grypus*) from Birth to Weaning. *Marine Ecology vol. 9, P181-189*

Stearns, S.C. and Hoekstra, R.F. (2005) Evolution: an introduction. *2nd Edition, Oxford University Press Inc, New York, USA*

Stewart, J.E., Pomeroy, P.P., Duck. C.D. and Twiss, S.D. (2014) Individual scale habitat suitability modelling provides evidence that lactating grey seals (*Halichoerus grypus*) prefer access to fresh water in order to drink. *Marine Mammal Science, in press.*

Strathearn, L., Fonagy, P., Amico, J. and Montague, P. R. (2009) Adult attachment predicts maternal brain and oxytocin response to infant cues. *Neuropsychopharmacology vol 34, No. 13, P2655-2666*

Szeto, A., McCabe, P., Nation, D.A., Tabak, B.A., Rossetti, M.A., McCullough, M.E., Schneiderman, N. and Mendez, A.J. (2011) Evalutaion of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin. *Psychosomatic Medicine vol 73, No. 5, P393-400*

Taylor, S.E., Saphire-Bernstein, S. and Seeman T.E. (2010) Are plasma oxytocin in women and plasma vasopressin in men biomarkers of distressed pair-bond relationships? *Psychological Science vol 21, No. 1, P3-7*

Tedman, R.A. and Bryden, M.M. (1979) Cow-pup behaviour of the Weddell seal, *Leptonychotes weddelli* (Pinnipedia), in Mcmurdo Sound, Antarctica. *Wildlife Reasearch vol 6, No. 1, P19-37*

Thornton, S., Davison, J.M. and Baylis, P.H., (1990) Effect of human pregnancy on metabolic clearance rate of oxytocin. *American Journal of Physiology – Regulatory, Intergrative and Comparative Physiology vol 259, No. 1, P21-24*

Tops, M., van Peer, J.M., Korf, J., Wijers, A.A. and Tucker, D.M. (2007) Aniety, cortisol and attachment predict plasma oxytocin. *Psychophysiology vol 44, No. 3, P444-449*

Tops, M., Bulsman-Pijlman, F.T., Boksem, M.A., Wijers, A.A. and Korf, J. (2012) Cortisol-induced increases of plasma oxytocin levels predict decreased immediate free recall of unpleasant words. *Frontiers in Psychiatry vol 16, No. 3, P43*

Trillmich, F. (2010) Parental Care: Adjustments to Conflict and Cooperation. *In Kappeler, P. ed. Animal Behaviour: Evolution and Mechanisms. Springer-Verlag, Berlin, Germany P267-298*

Tripovich, J.S., Charrier,I., Rogers, T.L., Canfield, R. and Arnould, J.P.Y. (2008) Acoustic features involved in the neighbour-stranger vocal recognition process in male Australian fur seals. *Behvaioural Processes vol 79. No. 1, P74-80*

Tsujimoto, M. and Hattori, A. (2005) The oxytocinase subfamily of M1 aminopeptidases. *Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics col 1751, No. 1, P9-18.*

Twiss, S.D. (1991) Behavioural and energetic determinants of individual mating success in male grey seals (*Halichoerus grypus*). *PhD thesis, the University of Glasgow, Scotland.*

Twiss, S.D., Pomeroy, P.P. and Anderson, S.S. (1994) Dispersion and site fiedelity of breeding male grey seals (*Halichoerus grypus*) on North Rona, Scotland. *Journal of Zoology vol. 233, No. 4, P683-693*

Twiss, S.D., Anderson, S.S. and Monaghan, P. (1998) Limited intra-specific variation in male grey seal (*Halichoerus grypus)* dominance relationships in relation to variation in male mating success and female availiability. *Journal of Zoology vol 246, P359-267*

Twiss, S.D., Caudron, A., Pomeroy, P.P., Thomas, C.J. and Mills, J.P. (2000) Finescale topographical correlates of behavioural investment in offspring by female grey seals, *Halichoerus grypus. Animal Behaviour vol. 59, No. 2, P327-338*

Twiss, S.D., Wright, N.C., Dunstone, N., Redman, P., Moss, S. and Pomeroy, P.P. (2002) Behavioural evidence of thermal stress from overheating in UK breeding gray seals. *Marine Mammal Science, vol 18, No. 2, P455-468*

Twiss, S.D., Duck, C. and Pomeroy, P.P. (2003) Grey seal pup mortality not explained by local breeding density on North Rona, Scotland. *Journal of Zoology vol 259, No. 1, PP83-91*

Twiss, S.D. and Franklin, J. (2010) Individually consistent behavioural patterns in wild, breeding male grey seals *(Halicherus grypus*). *Aquatic mammals vol 36, P234-238*

Twiss, S.D., Culloch, R. and Pomeroy, P.P. (2012a) An in-field experimental test of pinniped behavioural types. *Marine Mammal Science vol 28, No. 3, P280-294*

Twiss, S.D., Cairns, C., Culloch, R.M., Richards, S.A. and Pomeroy, P.P (2012b) Variation in female grey seal (*Halichoerus grypus)* reproductive performance correlates to proactive-reactive behavioural types. *PLOS one vol 7, No. 11, e49598*

Uvnäs-Moberg, K., Stock, S., Eriksson, M., Linden, A., Einarsson, S. and Kunavongkrit, A. (1985) Plasma levels of oxytocin increase in response to suckling and feeding in dogs and sows. *Acta Physiologica Scandinavica vol 124, No. 3, P391-398*

Uvnäs-Moberg, K. (1998) Oxytocin may mediate the benefits of positive social interaction and emotions. *Psychoneuroendocrinology vol 23, No. 8, P819-835*

van de Post, J.A.M., van Buul, B.J.A., Hart, A.A.M., van Heerikhuize, J.J., Pesman, G., Legros, J.J., Steegers, E.A.P., Swaab, D.F. and Boer, K. (1997)

Vasopressin and oxytocin levels during normal pregnancy: effects of chronic dietary sodium restriction. *Journal of Endocrinology vol 152, P345-354*

Varo N., Nuzzo R., Natal C., Libby P. and Schönbeck U. (2006) Influence of pre-analytical and analytical factors on soluble CD40L measurements. *Clinical Science vol 111, P341-347*

Viero, C., Shibuya, I., Kitamura, N., Verkhratsky, A., Fujihara, H., Katoh, A., Ueta, Y., Zingg, H.H., Chvatal, A., Sykova, E. and Dayanithi, G. (2010) Oxytocin: crossing the bridge between basic science and pharmacotherapy. *CNS Neuroscience and Therapeutics vol 16, No.5, P138-156*

Walker, W. (2005) The strengths and weaknesses of research designs involving quantitative measures. *Journal of Research into Nursing vol 10, No. 5, P571-582*

Wang, Z.X., Ferris, C.F. and De Vries, G. (1994) Role of septal vasopressin innervation in paternal behaviour in prairie voles (*Microtus ochrogaster). Proceedings of the National Academy of Science of the United States of America vol 91, No. 1, P400-404*

Wang, Z. and Young, L.J. (1997) Ontogeny of oxytocin and vasopressin receptor binding in the lateral septum in prairie and montane voles. *Developmental Brain Research vol 104, No. 1-2, P191-195*

Weisman, O., Zagoory-Sharon, O. and Feldman, R. (2012a) Oxytocin administration to parent enhances infant physiological and behavioural readiness for social engagement. *Biological Psychiatry vol 72, No.12, P982-989*

Weisman, O., Zagoory-Sharon, O. and Feldman, R. (2012b) Intranasal oxytocin administration is reflected in human saliva. *Psychoneuroendocrinology vol 37. No. 9, P1582-1596*

Weisman O., Zagoory-Sharon O., Schneiderman I., Gordon I. and Feldman R. (2013a) Plasma oxytocin distributions in a large cohort of women and men and their gender-specific associations with anxiety. *Psychoneuroendocrinology vol 38, No. 5, 694-701*

Weisman O., Delaherche, E., Rondeau, M., Chetouani, M., Cohen, D. and Feldman, R. (2013b) Oxytocin shapes parental motion during father-infant interaction. *Biology Letters vol 9, No. 6, 20130828.*

Wingfield, J.C., Hegner, R.E., Dufty Jr, A.M. and Ball, G.F. (1990) The challenge hypothesis: theoretical implications for patterns of testosterone secretion mating systems, and breeding strategies. *American Naturalist vol 136, No. 6, P829-846*

Winslow, J.T., Noble, P.L., Lyons, C.K., Sterk, S.M. and Insel, T.R. (2003) Rearing effects on cerebrospinal fluid oxytocin concentration and social buffering in rhesus monkeys. *Neuropsychopharmacology vol 28, No. 5, P910-918*

Wolf, J. and Trillmich, F. (2007) Beyond habitat requirements: individual fine-scale site fidelity in a colony of the Galapagos sea lion (*Zalophus wollebaeki*) creates conditions for social structuring. *Oecologia, vol 152, No. 3, P553-567*

Wood S. (2006a) Generalized Additive Models: An introduction with R. *Chapman and Hall/CRC*

Wood S. (2006b) Low-rank scale-invariant tensor product smooths for generalized additive mixed models. Biometrics 62: 1025-1036.

Wood S. (2011) Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. Journal of the Royal Statistical Society B 73: 3-36.

Wood S. (2012) mgcv: Mixed GAM Computation Vehicle with GCV/AIC/REML smoothness estimation. http://cran.r-project.org/web/packages/mgcv/index.html

Yayou K., Ito S., Yamamoto N., Kitagawa S. and Okamura H. (2010) Relationship of stress responses with plasma oxytocin and prolactin in heifer calves. *Physiology and Behaviour vol 99, No. 3, P362-369.*

Ylikorkala, O., Kauppila, A., Kivinen, S. and Viinikka, L. (1984) Treatment of inadequate lactation with oral sulpiride and buccal oxytocin. *Obstetrics and Gynecology vol 63, No. 1, P57-60*

Young, L.J., Winslow, J.T., Wang, Z., Gingrich, B., Guo, Q., MAtzuk, M.M. and Insel, T.R. (1997) Gene targeting approaches to neuroendocrinology: Oxytocin, maternal behaviour and affiliation. *Hormones and Behaviour vol 31, No. 3, P221-231*

Zak, P.J., Stanton, A.A. and Ahmadi, S. (2007) Oxytocin increases generosity in humans. *PLoS ONE vol. 2, No. 11, P1128-1133*

Appendices

Appendix A – Appendix for Chapter 5.

Table A-1. GAM and GLM variable selection for the six models of the behaviour displayed by mothers with dependant pups.

Variable	Levels	Retained in the final model?					
		Frequency of resting	**Frequency of 'awareness of pup' behaviours**	**Frequency of 'pup care' behaviours**	**Frequency of 'aggression' behaviours**	**Frequency of 'alertness' behaviours**	**Mother-pup distance**
Plasma oxytocin concentration in mothers (pg/ml)	Continuous data	Yes	Yes	Yes	Yes	Yes	Yes
Plasma oxytocin concentration in pups (pg/ml)	Continuous data	Yes	No	No	Yes	No	No
Mother Identity	Random effect smooth	Yes	Yes	Yes	Yes	Yes	No
Pup Sex	Male/female	Yes	Yes	No	Yes	No	No
Time during lactation	Early/late	Yes	No	Yes	Yes	Yes	Yes
Pup birth date	Continuous data	Yes	No	No	Yes	No	No
Rate of mass lost from mother (kg/day)	Continuous data	Yes	No	No	Yes	No	No
Rate of mass gain in pup (kg/day)	Continuous data	Yes	No	No	Yes	No	No
Time within 1 body length	Continuous data	Yes	Yes	No	Yes	No	No

Table A-2. GAM variable selection for the five models of the behaviour displayed by dependant pups while with their mothers.

Variable	**Levels**	**Retained in the final model?**				
		Frequency of resting	**Frequency of 'interaction with mother' behaviours**	**Frequency of 'pup activity' behaviours**	**Frequency of 'play' behaviours**	**Frequency of 'awareness of mother' behaviours**
Plasma oxytocin concentration in mothers (pg/ml)	Continuous data	Yes	No	Yes	No	No
Plasma oxytocin concentration in pups (pg/ml)	Continuous data	Yes	Yes	Yes	Yes	Yes
Mother Identity	Random effect smooth	Yes	Yes	Yes	Yes	Yes
Pup Sex	Male/female	Yes	No	No	Yes	No
Time during lactation	Early/late	Yes	No	Yes	Yes	Yes
Pup birth date	Continuous data	Yes	No	No	Yes	No
Rate of mass lost from mother (kg/day)	Continuous data	Yes	Yes	No	No	No
Rate of mass gain in pup (kg/day)	Continuous data	Yes	No	No	Yes	No
Time within 1 body length	Continuous data	Yes	Yes	Yes	No	No

Table A-3. GAM variable selection for the two models of plasma oxytocin concentrations in dependant pups and mothers.

Variable	**Levels**	**Retained in the final model?**	
		Maternal plasma oxytocin concentrations	**Pup plasma oxytocin concentrations**
Plasma oxytocin concentration in mothers (pg/ml)	Continuous data	NA	Yes
Plasma oxytocin concentration in pups (pg/ml)	Continuous data	Yes	NA
Mother Identity	Random effect smooth	Yes	Yes
Pup Sex	Male/female	Yes	No
Time during lactation	Early/late	Yes	No
Pup birth date	Continuous data	Yes	No
Rate of mass lost from mother (kg/day)	Continuous data	Yes	Yes
Rate of mass gain in pup (kg/day)	Continuous data	Yes	Yes
Time within 1 body length	Continuous data	Yes	No

Table A-4. All model variables from the five GAMs and one GLM for frequency of the five behavioural components and the mother-pup distance in grey seal mothers, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Resting	Maternal plasma oxytocin concentrations (pg/ml)	-0.05	0.3	0.8
	Pup plasma oxytocin concentrations (pg/ml)	-0.07	0.08	0.4
	Pup sex	2.2	1.6	0.2
	Time during lactation	-5.3	1.5	0.001
	Pup birth date	-0.02	0.2	0.9
	Rate of maternal mass loss (kg/day)	-1.6	1.2	0.2
	Rate of pup mass gain (kg/day)	2.7	2.6	0.3
	Mother-pup distance (adult body lengths)	-0.2	0.5	0.7
Awareness of pup behaviour	Maternal plasma oxytocin concentrations (pg/ml)	0.04	0.01	0.01
	Pup sex	-0.3	0.1	0.01
	Mother-pup distance (adult body lengths)	0.05	0.03	0.1
Pup care behaviour	Maternal plasma oxytocin concentrations (pg/ml)	-0.02	0.02	0.3
	Time during lactation	0.4	0.1	0.009
Aggression behaviour	Maternal plasma oxytocin concentrations (pg/ml)	0.004	0.08	0.9
	Pup plasma oxytocin concentrations (pg/ml)	0.03	0.03	0.2
	Pup sex	0.3	0.5	0.6
	Time during lactation	0.1	0.1	0.3
	Pup birth date	-0.04	0.06	0.5
	Rate of maternal mass loss (kg/day)	0.7	0.5	0.1
	Rate of pup mass gain (kg/day)	-0.4	0.8	0.7
	Mother-pup distance (adult body lengths)	0.1	0.1	0.3
Alertness behaviour	Maternal plasma oxytocin concentrations (pg/ml)	-0.04	0.06	0.5
	Time during lactation	0.6	0.4	0.1
Mother-pup distance	Maternal plasma oxytocin concentrations (pg/ml)	-0.09	0.05	0.05
	Time during lactation	-0.8	0.3	0.02
Maternal Plasma Oxytocin	Pup plasma oxytocin concentrations (pg/ml)	0.02	0.006	0.002
	Pup sex	0.1	0.2	0.3
	Time during lactation	-0.1	0.1	0.2
	Pup birth date	0.01	0.02	0.4
	Rate of maternal mass loss (kg/day)	0.06	0.09	0.5

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Maternal Plasma Oxytocin	Rate of pup mass gain (kg/day)	0.2	0.2	0.4
	Mother-pup distance (adult body lengths)	-0.07	0.04	0.09

Table A-5. Random effect variables from the five GAMs for the five behavioural components and the mother-pup distance in grey seal mothers, their standard deviations and p values.

Dependant variable	GAMM random effect	Standard deviation	P value
Resting	Mother identity	1.04	0.9
Awareness of pup behaviour	Mother identity	0.03	0.6
Pup care behaviour	Mother identity	0.09	0.4
Aggression behaviour	Mother identity	0.0000005	NA
Alertness behaviour	Mother identity	0.7	0.2
Maternal Plasma Oxytocin	Mother identity	0.6	0.06

Table A-6. All model variables from the five GAMs for the five behavioural components in dependant grey seal pups, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Resting	Maternal plasma oxytocin concentrations (pg/ml)	0.7	0.6	0.3
	Pup plasma oxytocin concentrations (pg/ml)	0.05	0.2	0.8
	Pup sex	-2.1	3.5	0.6
	Time during lactation	2.7	3.5	0.4
	Pup birth date	-0.6	0.4	0.1
	Rate of maternal mass loss (kg/day)	-5.9	2.7	0.04
	Rate of pup mass gain (kg/day)	8.7	5.7	0.1
	Mother-pup distance (adult body lengths)	-0.003	1.2	0.9
Interaction with mother behaviour	Pup plasma oxytocin concentrations (pg/ml)	0.005	0.008	0.5
	Rate of maternal mass loss (kg/day)	0.6	0.1	<0.001
Pup activity behaviour	Maternal plasma oxytocin concentrations (pg/ml)	-0.1	0.04	0.003
	Pup plasma oxytocin concentrations (pg/ml)	-0.006	0.009	0.5
	Time during lactation	-0.3	0.1	0.05
	Mother-pup distance (adult body lengths)	0.08	0.04	0.05
Play behaviour	Pup plasma oxytocin concentrations (pg/ml)	0.2	0.08	0.02
	Pup sex	-3.1	1.8	0.09
	Time during lactation	-3.9	1.4	0.007
	Pup birth date	0.5	0.2	0.02
	Rate of pup mass gain (kg/day)	-3.9	2.6	0.1
Awareness of mother behaviour	Pup plasma oxytocin concentrations (pg/ml)	-0.006	0.009	0.5
Pup plasma oxytocin	Maternal plasma oxytocin concentrations (pg/ml)	0.06	0.01	<0.001
	Rate of maternal mass loss (kg/day)	-0.2	0.07	0.005
	Rate of pup mass gain (kg/day)	0.3	0.2	0.09

Table A-7. Random effect variables from the five GAMs for the five behavioural components in dependant grey seal pups, their standard deviations and p values.

Dependant variable	GAMM random effect	Standard deviation	P value
Resting	Mother identity	0.0000000004	NA
Interaction with mother behaviour	Mother identity	0.09	0.1
Pup activity behaviour	Mother identity	0.07	0.9
Play behaviour	Mother identity	3.1	0.9
Awareness of mother behaviour	Mother identity	0.08	0.7
Pup plasma oxytocin	Mother identity	0.2	<0.001

Appendix B – Appendix for Chapter 7.

Table B-1. GAM variable selection for the four models of behavioural change during oxytocin manipulations on newly weaned grey seal pups in a semi-wild environment.

Variable	**Levels**	**Retained in the final model?**			
		Frequency of approaches	**Frequency of investigations**	**Frequency of interactions**	**Time within 1 body length**
Manipulation	OT1, OT2, Sal1, Sal2	Yes	Yes	Yes	Yes
Sex	Male/female	No	No	No	No
Pup Identity	Random effect smooth	Yes	Yes	Yes	Yes
Approaches	Frequency count	NA	Yes	Yes	No
Investigations	Frequency count	No	No	Yes	No
Aggression	Frequency count	Yes	Yes	NA	Yes
Time within 1 body length	Continuous data	Yes	No	Yes	NA
Time spent in captivity	Continuous data	No	No	Yes	Yes

Table B-2. GAM variable selection for the five models of behavioural change during oxytocin manipulations on free ranging mother grey seals with dependant pups.

Variable	**Levels**	**Retained in the final model?**				
		Frequency of investigations	**Frequency of interactions**	**Time within 1 body length**	**Time in physical contact**	**Time nursing**
Manipulation	OT1, OT2, Sal1, Sal2	Yes	Yes	Yes	Yes	Yes
Colony	Isle of May/North Rona	Yes	Yes	Yes	Yes	Yes
Mother Identity	Random effect smooth	Yes	Yes	Yes	Yes	Yes
Investigations	Frequency count	NA	Yes	No	Yes	Yes
Interactions	Frequency count	Yes	NA	No	Yes	Yes
Alert	Frequency count	Yes	No	No	Yes	Yes
Aggression	Frequency count	Yes	No	No	Yes	Yes
Time within 1 body length	Continuous data	Yes	Yes	NA	Yes	Yes
Time in physical contact	Continuous data	Yes	Yes	Yes	NA	Yes
Time nursing	Continuous data	Yes	Yes	No	Yes	NA
Time resting	Continuous data	Yes	No	No	Yes	Yes

Table B-3. All model variables from the four GAMs for frequency of approach, investigative, aggressive behaviour and the time spent within one body length for manipulated newly weaned pups, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Approaches	Trial type (Sal1)	-0.4	1.6	0.02
	Trial type (Sal2)	-0.5	0.1	<0.001
	Trial type (OT2)	-0.4	1.5	0.01
	Time spent within 1 body length	0.0001	0.00008	0.15
	Investigative behaviour	0.03	0.003	<0.001
	Aggressive interactions	-0.01	0.003	<0.001
Investigative	Trial type (Sal1)	13.9	4.6	0.003
	Trial type (Sal2)	1.9	4.5	0.67
	Trial type (OT2)	2.5	4.7	0.6
	Approaches	1.9	0.3	<0.001
	Aggressive interactions	0.5	0.08	<0.001
Aggressive interactions	Trial type (Sal1)	-0.6	0.2	<0.001
	Trial type (Sal2)	0.2	0.09	0.08
	Trial type (OT2)	-0.4	0.2	0.005
	Time spent within 1 body length	0.0009	0.00005	<0.001
	Approaches	-0.03	0.008	<0.001
	Investigative behaviour	0.02	0.002	<0.001
	Time in captivity	-0.1	0.02	<0.001
Time spent within 1 body length	Trial type (Sal1)	-753.9	233.8	0.001
	Trial type (Sal2)	-153.4	233.7	0.5
	Trial type (OT2)	-126.2	242.1	0.6
	Aggressive interactions	7.1	3.9	0.08
	Time in captivity	84.7	37.9	0.03

Table B-4. Random effect variables from the four GAMs for frequency of approach, investigative, aggressive behaviour and the time spent within one body length for manipulated newly weaned pups, their standard deviations and p values.

Dependant variable	GAMM random effect	Standard deviation	P value
Approaches	Focal Individual	0.4	0.02
	Response Individual	0.3	0.3
Investigative	Focal Individual	7.6	0.3
	Response Individual	0.05	NA
Aggressive interactions	Focal Individual	1.2	<0.001
	Response Individual	1.3	<0.001
Time spent within 1 body length	Focal Individual	255.5	0.8
	Response Individual	115.3	0.9

Table B-5. All model variables from the five GAMs for frequency of pup checks, interactions with pup, the time spent within one body length, time spent in contact with the pup and time spent nursing the pup in manipulated free ranging mothers, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Check pup	Trial type (Sal1)	0.0	0.0	NA
	Trial type (Sal2)	-15.8	11.5	0.2
	Trial type (OT2)	-17.2	11.4	0.2
	Colony	5.0	11.4	0.7
	Time spent within 1 body length	-0.005	0.003	0.1
	Time spent in contact	-0.008	0.004	0.09
	Time spent nursing	-0.006	0.006	0.3
	Time spent resting	-0.007	0.005	0.2
	Frequency of interactions	0.9	0.3	0.02
	Frequency of alert behaviours	0.8	0.2	0.003
	Frequency of aggressive behaviours	-0.6	0.4	0.2
Interactions	Trial type (Sal1)	-0.7	1.4	0.6
	Trial type (Sal2)	0.0	0.0	NA
	Trial type (OT2)	-0.7	1.0	0.5
	Colony	-0.4	1.2	0.7
	Time spent within 1 body length	0.001	0.0007	0.07
	Time spent in contact	0.0004	0.0004	0.3
	Time spent nursing	0.002	0.0006	<0.001
	Frequency of pup checks	0.8	0.02	<0.001
Time spent within 1 body length	Trial type (Sal1)	0.0	0.0	NA
	Trial type (Sal2)	-1075.7	569.5	0.07
	Trial type (OT2)	-630.5	560.5	0.3
	Time spent in contact	0.8	0.2	0.002
Time spent in contact	Trial type (Sal1)	0.0	0.0	NA
	Trial type (Sal2)	-82.1	436.2	0.9
	Trial type (OT2)	-248.5	412.8	0.6
	Colony	-367.3	343.9	0.3
	Time spent within 1 body length	0.3	0.1	0.04
	Time spent nursing	-0.4	0.3	0.3
	Time spent resting	-0.5	0.2	0.03
	Frequency of pup checks	-15.4	10.6	0.2
	Frequency of interactions	21.9	14.2	0.15
	Frequency of alert behaviours	15.9	11.4	0.2
	Frequency of aggressive behaviours	-34.3	14.6	0.04
Time spent nursing	Trial type (Sal1)	-84.6	368.2	0.8
	Trial type (Sal2)	0.0	0.0	NA
	Trial type (OT2)	-262.9	346.1	0.5
	Colony	-322.4	289.6	0.3
	Time spent within 1 body length	0.08	0.1	0.6
	Time spent in contact	-0.3	0.3	0.3

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
	Time spent resting	-0.2	0.2	0.2
	Frequency of pup checks	0.3	9.6	0.9
	Frequency of interactions	12.0	12.6	0.4
	Frequency of alert behaviours	9.2	10.0	0.4
	Frequency of aggressive behaviours	-25.5	12.9	0.07

Table B-6. Random effect variables from the five GAMs for frequency of pup checks, interactions with pup, the time spent within one body length, time spent in contact with the pup and time spent nursing the pup manipulated free ranging mothers, their estimates, standard errors and p values., their standard deviations and p values.

Dependant variable	GAMM random effect	Standard deviation	P value
Check pup	Mother identity	11.2	0.3
Interactions	Mother identity	1.5	0.9
Time spent within 1 body length	Mother identity	5.2	NA
Time spent in contact	Mother identity	1.6	NA
Time spent nursing	Mother identity	1.3	NA

Appendix C – Appendix for Chapter 8

Table C-1. GAM variable selection for the four models of behavioural change during social recognition trials on newly weaned grey seal pups in a semi-wild environment.

Variable	**Levels**	**Retained in the final model?**			
		Frequency of approaches	**Frequency of investigations**	**Frequency of aggressive interactions**	**Time within 1 body length**
Trial type	Stranger/familiar	Yes	Yes	Yes	Yes
Sex	Male/female	No	No	No	No
Trial Individual 1 Identity	Random effect smooth	Yes	Yes	Yes	Yes
Trial Individual 2 Identity	Random effect smooth	Yes	Yes	Yes	Yes
Approaches	Frequency count	NA	Yes	Yes	Yes
Investigations	Frequency count	Yes	No	Yes	Yes
Aggression	Frequency count	Yes	Yes	NA	Yes
Time within 1 body length	Continuous data	No	Yes	Yes	NA
Time spent in captivity	Continuous data	No	Yes	Yes	No

Table C-2. All model variables from the four GAMs for frequency of approach, investigative, aggressive behaviour and the time spent within one body length for social recognition trials, their estimates, standard errors and p values.

Dependant variable	Explanatory variable	Estimate	Standard Error	P value
Approaches	Trial type (Stranger)	0.1	0.1	0.2
	Investigative behaviour	-0.008	0.002	<0.001
	Aggressive interactions	0.01	0.001	<0.001
Investigative	Trial type (Stranger)	0.09	0.03	<0.001
	Time spent within 1 body length	0.0002	0.00003	<0.001
	Approaches	0.04	0.002	<0.001
	Aggressive interactions	0.004	0.0005	<0.001
	Time in captivity	-0.02	0.004	<0.001
Aggressive interactions	Trial type (Stranger)	0.4	0.05	<0.001
	Time spent within 1 body length	0.0003	0.00006	<0.001
	Approaches	-0.05	0.007	<0.001
	Investigative behaviour	0.01	0.0009	<0.001
	Time in captivity	-0.02	0.007	0.005
Time spent within 1 body length	Trial type (Stranger)	-22.8	131.7	0.9
	Approaches	10.3	17.3	0.6
	Investigative behaviour	5.3	2.5	0.03
	Aggressive interactions	1.7	3.1	0.6

Table C-3. Random effect variables from the four GAMs for frequency of approach, investigative, aggressive behaviour and the time spent within one body length for social recognition trials, their standard deviations and p values.

Dependant variable	GAMM random effect	Standard deviation	P value
Approaches	Focal Individual	0.6	<0.001
	Response Individual	0.1	0.7
Investigative	Focal Individual	0.3	<0.001
	Response Individual	0.3	<0.001
Aggressive interactions	Focal Individual	0.6	<0.001
	Response Individual	0.9	<0.001
Time spent within 1 body length	Focal Individual	441.9	0.04
	Response Individual	418.2	0.05

Journal of Neuroscience Methods 226 (2014) 73–79

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Validation of an enzyme-linked immunoassay (ELISA) for plasma oxytocin in a novel mammal species reveals potential errors induced by sampling procedure☆

Kelly J. Robinson[a,*], Neil Hazon[b], Mike Lonergan[a], Patrick P. Pomeroy[a]

[a] Sea Mammal Research Unit, Scottish Oceans Institute, University of St Andrews, St Andrews, Fife KY16 8LB, UK
[b] Scottish Oceans Institute, University of St Andrews, Scotland KY16 8LB, UK

HIGHLIGHTS

- An ELISA protocol for plasma oxytocin in phocid seals was validated.
- Sample handling protocols for accurately detecting plasma oxytocin were compared.
- Concentrations detected in raw plasma varied significantly with vacutainer type.
- Concentrations detected in extracted plasma were unaffected by vacutainer type.
- Capture and restraint protocol affected concentrations detected in raw plasma.

ARTICLE INFO

Article history:
Received 23 November 2013
Received in revised form 17 January 2014
Accepted 20 January 2014

Keywords:
Oxytocin
EDTA
Heparin
Extraction
Plasma
Seal

ABSTRACT

Background: The neuropeptide oxytocin is increasingly the focus of many studies investigating human and animal social behaviours and diseases. However, interpretation and comparison of results is made difficult by a lack of consistent methodological approaches towards analysing this hormone.
New method: This study determined the sample collection and analysis protocols that cause the least amounts of protocol dependant variation in plasma oxytocin concentrations detected by ELISA. The effect of vacutainer type, sample extraction prior to analysis and capture and restraint protocol were investigated while validating an assay protocol for two novel species, grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*).
Results: Where samples are extracted prior to analysis, vacutainer type (EDTA mean: 8.25 ± 0.56 pg/ml, heparin mean: 8.25 ± 0.62 pg/ml, $p = 0.82$), time taken to obtain a sample and restraint protocol did not affect the concentration of oxytocin detected. However, concentrations of oxytocin detected in raw plasma samples were significantly higher than those in extracted samples, and varied significantly with vacutainer type (EDTA mean: 534.4 ± 43.7 pg/ml, heparin mean: 300.9 ± 19.6 pg/ml, $p < 0.001$) and capture and restraint methodology. There was no relationship between oxytocin concentrations detected in raw and extracted plasma ($p = 0.25$).
Comparison with existing method(s): Over half the reviewed published studies analysing plasma oxytocin use raw plasma and different vacutainer types are used without consistency or justification through-out the literature.
Conclusions: We caution that studies using raw plasma are likely to over estimate oxytocin concentrations, cannot be used to accurately infer true values via correlations and are susceptible to variation according vacutainer type.

1. Introduction

Oxytocin is a neuropeptide crucial for initiating and modulating maternal and social behaviour across vertebrate animals (Gimpl and Fahrenholz, 2001). Produced in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus, oxytocin is stored in the posterior pituitary gland until its release is triggered by a variety of stimuli, including scent, sight, touch and sound of a dependant infant or social partner (Uvnäs-Moberg, 1998;

* Corresponding author. Tel.: +44 01334462635.
E-mail addresses: kjr33@st-andrews.ac.uk, kelly.j.robinson@hotmail.co.uk (K.J. Robinson).

0165-0270/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneumeth.2014.01.019

Neumann, 2008; MacDonald and MacDonald, 2010). Oxytocin is released both centrally into the brain and peripherally into circulation (Neumann and Landgraf, 2012) and it promotes care-giving and pro-social behaviours (Kosfeld et al., 2005; Ross and Young, 2009) by providing a physiological reward for their expression by activating reward and pleasure pathways in the brain (Baskerville and Douglas, 2010).

While its role in the physiology of parturition and lactation has long been known (Dale, 1906), oxytocin's effects on behaviour are still being explored. Studies looking to investigate peripheral levels of the hormone in relation to both animal and human behaviour have increased throughout the 21st century. In recent years there have been many studies examining possible links between oxytocin and a range of human psychological conditions, their causes and treatment including post-partum depression (Levine et al., 2007; Feldman et al., 2010a; Skrundz et al., 2011), anxiety (Hoge et al., 2012; Weisman et al., 2013), autism (Modahl et al., 1998; Hollander et al., 2003; El-Masry et al., 2010), depression (Scantamburlo et al., 2007; Holt-Lunstad et al., 2011), stress (Heinrichs et al., 2003; Grippo et al., 2009), anorexia nervosa (Hoffman et al., 2012) and suicidal behaviour (Deisenhammer et al., 2012). Oxytocin has also been used to investigate social and reproductive behaviour and physiology in a variety of domestic and laboratory mammalian species such as dogs (Odendaal and Meintjes, 2003), sheep (Lévy et al., 1995), primates (Amico et al., 1990; Maestripieri et al., 2009), mice (McCarthy, 1990), voles (Bales et al., 2007) and rats (Popik et al., 1992). However plasma oxytocin concentrations have never been determined in any mammalian species outside of laboratory, domestic or primate species, and the current study reports for the first time data from studies conducted on two species of phocis seals the grey seal (*Halichoerus grypus*) and harbour seal (*Phoca vitulina*).

Oxytocin has been assayed in various substrates including saliva (Carter et al., 2007; Feldman et al., 2010a,b), urine (Nagasawa et al., 2009; Moscovice and Ziegler, 2012; Crockford et al., 2013), cerebro-spinal fluid (Devarajan and Rusak, 2004; Martínez-Lorenzana et al., 2008), and milk (Leake et al., 1981; Prakash et al., 2009). However the most common medium used for oxytocin detection is plasma (of 50 papers reviewed using one or multiple substrates, $n = 39$ plasma, $n = 9$ saliva, $n = 8$ urine, $n = 6$ cerebro-spinal fluid, $n = 5$ serum and $n = 2$ milk). Despite its potential as a tool for investigating the expression of social and maternal behaviour in mammalian species, few analysis protocols for detecting oxytocin concentrations in plasma taken from wild populations or species have been validated.

The effect of capture and sampling procedure on detected plasma oxytocin concentrations of wild mammalian species has never been investigated in any prior study, and it is vital to ensure that abnormal concentrations of oxytocin are not being generated during this potentially stressful process. Oxytocin is documented to be released into circulation during extreme stressors such as restraint over an extended time period in rodents (Grippo et al., 2009; Pournajafi and Carter, unpublished observations) and is hypothesised to occur during procedures such as injections (Devarajan and Rusak, 2004). Therefore any attempt to study basal levels of this hormone in wild populations, which must be manually or chemically restrained during the sampling procedure, must be accompanied by investigation of whether oxytocin is released when sampling occurs.

Even with the increasing number of studies using plasma to investigate oxytocin, there is still contention about the appropriate way to collect and analyse samples accurately, and there has been a call for new research into reliable, accurate methodologies for investigators to utilise in future research (McCullough et al., 2013). The majority of studies published use an enzyme-linked immunosorbent assay (ELISA) kit manufactured by Assay Designs Inc. (Ann Arbor, MI, USA). However despite this manufacturer providing a protocol for collection and preparation prior to analysis, there is huge variation between studies in the methods researchers actually employ (of 39 papers, $n = 3$ unextracted serum, $n = 13$ extracted plasma, $n = 17$ unextracted plasma and $n = 6$ did not specify if extraction was used. For vacutainer type used in sampling, $n = 12$ ethylenediaminetetraacetic acid (EDTA) vacutainers, $n = 10$ lithium heparin vacutainers and $n = 14$ did not specify what vacutainer was used). In addition to the varying analysis protocols, there is disagreement in the literature over whether oxytocin concentrations measured in raw plasma are correlated with those in extracted samples, and how accurate the use of raw plasma is (McCullough et al., 2013). Currently some studies rely on the hypothesis that there is a direct relationship between oxytocin concentrations determined in extracted and raw samples (Hoge et al., 2012), provide data showing a correlation between oxytocin levels in raw and extracted serum samples (Michopoulos et al., 2011) or have validated oxytocin ELISAs for rodent species using raw plasma samples (Kramer et al., 2004). However, two papers to date have directly tested the benefits of extracting plasma samples prior to analysis, with both recommending the use of solid phase extraction with C18 Sep-Pak columns (Bachem, San Carlos, CA) as the best method to gain accurate detection levels for oxytocin in plasma (Szeto et al., 2011; Cool and DeBrosse, 2003). Szeto et al. (2011) report no correlation between oxytocin concentrations measured in raw plasma and those in extracted plasma, and call for further work to be carried out in order to develop reliable methods for oxytocin analysis. Additionally the large discrepancy between reported oxytocin concentrations detected in raw and extracted plasma (>100 pg/ml and <30 pg/ml, respectively, reviewed in Szeto et al., 2011) make comparisons across studies with different methodologies impossible and interpretation of which values most accurately reflect true oxytocin concentrations difficult.

This study investigates oxytocin plasma concentrations for the first time in two marine mammal species, the grey seal and harbour seal, and assesses how the measured concentration of oxytocin in plasma is affected by the type of anticoagulant used during collection, the handling process of the individual being sampled and subsequently whether the plasma is extracted prior to analysis. The relationship between raw and extracted oxytocin concentrations detected was also investigated in order to determine whether one can be used to accurately predict the other. This work was conducted during the development of the best protocol for detecting oxytocin accurately in phocid seals and was conducted as part of a larger study on phocid reproductive behaviour and physiology.

2. Methods

2.1. Ethics statement

This study used captive harbour and grey seals held under UK Home Office licence at the Sea Mammal Research Unit in St Andrews, Scotland. Capture and handling procedures were performed under Home Office project licence #60/4009 and conformed to the UK Animals (Scientific Procedures) Act, 1986. All research was approved ethically by the University of St Andrews Animal Welfare and Ethics Committee.

2.2. Study animals

Three seals provided blood samples for the comparison of vacutainer type and extraction protocol. Samples were collected over the period the seals were within the facility. The first was an adult male harbour seal of unknown age, brought into the facility on 10.02.11 from the Eden River, Scotland and released in November 2011. The other two were immature grey seals of known age, one male and one female. Both of these were born in November of the 2011 breeding season on the Isle of May, Scotland and brought

into captivity on 06.12.11 at approximately one month of age. The female was released in October 2012 and the male in December 2012. Additionally, a two-year old captive male harbour seal from the Moray Firth, Scotland kept from 01.10.10 until 29.10.11, was used to generate a dataset on the clearance rate of oxytocin in phocid seals using extracted and un-extracted samples.

2.3. Plasma sampling and analysis

Plasma samples were taken at approximately monthly intervals throughout the period of captivity ($n = 27$). A sample collected using heparin vacutainers was collected in all 27 sampling opportunities; however during two of the sampling opportunities it was not possible additionally to collect an EDTA sample, giving 25 matching EDTA plasma samples and a total number of 52 samples to compare to each other. To obtain a plasma sample, animals were captured and physically restrained, additionally animals over eight months of age were chemically immobilised using Zoletil® (Virbac). Samples were drawn from the extradural vein into either 10 ml lithium heparin or EDTA vacutainers without addition of aprotinin and stored on ice until they could be spun and frozen at −20 °C (CS Carter 2010 personal communication). While the ELISA protocol recommends freezing samples at −70 °C, this work was part of a larger project to determine the viability of samples obtained from a field site where only a −20 °C freezer was available. This study attempted to obtain all samples within five minutes of the initial disturbance to the animal as the reported short half life of oxytocin in plasma (Cool and DeBrosse, 2003) would suggest that slow sampling would not give a representative sample. The time taken to obtain a sample, and if chemical immobilisation was used, was recorded to account for these variables in the analysis. At each sampling event four 10 ml vacutainers of plasma (two of each type) were taken. Plasma was then analysed for oxytocin using an ELISA (Assay Designs Inc., Ann Arbor, MI, USA) with each sample undergoing solid-phase extraction using Sep-Pak C18 columns (Szeto et al., 2011; Cool and DeBrosse, 2003) prior to analysis following the manufacturer's instructions with the following modifications. First it was necessary to extract from 3 ml of plasma to allow use of the most sensitive part of the assay curve for determining concentrations of oxytocin. Second all samples had to be centrifuged after acidification for thirty minutes rather than fifteen; otherwise there was a risk that the plasma samples would block the Sep-Pak columns used to extract the peptide due to the high lipid and albumin concentrations present in phocid plasma (Hall, 1998). Extracts were then frozen until analysed, whereupon they were rehydrated according to the assay instructions and run on the ELISA plate. Both raw plasma and extracted plasma were analysed for all 52 samples collected during analysis, giving a total sample size of 104 samples for this study. The plate was read using a BioTek ELx800 reader and the standard curve and assay results for all plates were then fitted using the calibFit package (Haaland et al., 2011) in R version 2.9.2 (R Development Core Team, 2008). Recovery rates for the extraction and ELISA procedure were 107% ($n = 10$), intra-assay coefficient of variance for this assay was 4.6% and inter-assay coefficient of variance over the seven plates used in this study was 4%.

2.4. Clearance trial

For the duration of the 1 hour trial, the study animal was captured and chemically immobilised using Zoletil® (Virbac). A baseline sample was drawn from the extradural vein into 10 ml lithium heparin vacutainers, and then 3.96 μg oxytocin (0.022 ml of 0.18 mg/ml Oxytocin-S, Intervet UK Ltd. with 0.128 ml saline solution) was injected intravenously to create a hormone spike in the plasma. Serial samples were then taken every minute for the first five minutes of the trial, then every five minutes until 20 minutes post-injection, and finally every ten minutes until one hour post-injection. Samples were then stored, spun, frozen and analysed as described above, generating clearance curves for both extracted and raw versions of the same samples. The plasma clearance rate (CL) for both raw and extracted plasma samples from the same clearance trail were calculated using the following equation (Morin et al., 2008):

$$\mathrm{CL} = \frac{D}{\mathrm{AUC}}$$

where area under curve (AUC) is the area under the curve generated in the GAMs detailed below describing the relationship between plasma oxytocin concentrations and time post injection and D is the initial bolus of oxytocin injected in picograms (pg).

2.5. Statistical analysis

All analyses were performed using the statistical package R 2.15.0 (R Development Core Team, 2012). Generalised additive mixed models (GAM) (Wood, 2006a) were used to take into account variability in oxytocin generated by species, sex, the day of year the samples were taken, and the analysis protocol used (EDTA extracted, EDTA raw, heparin extracted and heparin raw). The models had a gamma error distribution with a log link and were fitted using the multiple generalised cross validation library mgcv (Wood, 2012). As any variation with 'day of year' (DOY) is unlikely to be linear, this was fitted as a smooth function (Wood, 2006b). Differences between species and sex in the seasonal pattern were examined by fitting additional smooth functions. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). All other variables were fitted as fixed effects. Backwards stepwise elimination was carried out by using the Akaike information criterion (AIC) to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models. On this basis, all smooths were removed from the model, and DOY was removed entirely, simplifying the model into a generalised linear model (GLM) and leaving three fixed effects (species, sex and analysis protocol) which produced the best performing model.

Two separate GLMs were used to investigate any relationship between time taken to obtain a plasma sample, the use of chemical immobilisation or physical restraint and vacutainer type on the oxytocin concentrations detected in raw and extracted plasma. This could not be included in the first GLM as some values were missing from one individual's data, which would have reduced the number of samples included in the analysis above. The time in minutes it took to obtain a plasma sample from the initial disturbance to the individual, the type of vacutainer used for sample collection, what form of restraints (physical or chemical) were used for the procedure and an interaction term for sampling time/restraint type were fitted as fixed effects and a gamma error distribution with a log link was used. Backwards stepwise elimination was carried out by using the Akaike information criterion (AIC) to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models.

To investigate any correlations between oxytocin concentrations detected in extracted and raw plasma samples ($n = 52$), two GLM was used to model the relationship between the two measures taken with heparin ($n = 27$) and EDTA ($n = 25$) vacutainers separately and to predict extracted values of oxytocin from concentrations detected in raw samples. All GLM fits were fitted with gamma error distributions with log links and were checked with diagnostic plots and AIC scores to determine the best fits for the data.

Non-linear and GAM approaches were investigated to quantify the clearance curves generated from the IV oxytocin injection. The GAM approach was determined to be the most suitable for comparing the quality of the two clearance curves, and GAMs were made

Table 1
Fixed effect variables from the GLM of the effect of analysis protocol on detected oxytocin concentration, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Analysis protocol (heparin extracted)	0.03	0.087	0.71
Analysis protocol (heparin raw)	3.66	0.087	<0.001
Analysis protocol (EDTA raw)	4.21	0.088	<0.001
Sex	−0.18	0.072	0.012
Species	−0.53	0.077	<0.001

Table 2
Mean concentrations of oxytocin detected (pg/ml) in phocid plasma according to vacutainer type used to collect the sample and extraction protocol with standard errors.

	EDTA vacutainers	Heparin vacutainers
Extracted plasma	8.1 ± 0.6	8.3 ± 0.66
Raw plasma	543.2 ± 43.6	300.9 ± 19.6

for both extracted results and raw results. Both used one fixed effect (minutes post oxytocin injection) and as this variable was not linear it was fitted as a smooth function (Wood, 2006b) with smoothing parameters set by maximum likelihood (Wood, 2011).

3. Results

3.1. Causes of variability in oxytocin detection in seals

Of the variables tested, the GLM model showed that analysis protocol ($n = 104$ GLM, $F_{3,101} = 934.8$, $p < 0.001$) sex ($n = 104$ GLM, $F_{1,103} = 0.011$, $p = 0.01$) and species ($n = 104$ GLM, $F_{1,103} = 46.9$, $p < 0.001$) significantly affected the oxytocin detected in samples (Table 1). There was no significant difference between extracted samples taken with EDTA (mean = 8.1 ± 0.6 pg/ml) or heparin (mean = 8.3 ± 0.6 pg/ml) vacutainers ($p = 0.71$). However there were significant differences between all extracted and raw plasma protocols regardless of vacutainer type ($p < 0.001$) and between samples analysed raw taken using EDTA (mean = 543.2 ± 43.6 pg/ml) and heparin (mean = 300.9 ± 19.6 pg/ml) vacutainers ($p < 0.001$) (Table 2).

3.2. The effect of sampling time and restraint protocol on oxytocin detected

No relationship was found between oxytocin concentration detected in extracted plasma and the use of chemical or physical restraint in the capture process ($n = 36$ GLM, $F_{1,35} = 0.73$, $p = 0.27$), time taken to obtain a sample from first contact ($n = 36$ GLM, $F_{1,35} = 1.03$, $p = 0.14$), the interaction term for time to sample and type of restraint ($n = 36$ GLM, $F_{1,35} = 2.41$, $p = 0.13$) or vacutainer type ($n = 36$ GLM, $F_{1,35} = 0.61$, $p = 0.39$) (Table 3).

There were significant differences in detected oxytocin concentrations in raw plasma with the time taken to obtain a sample from first contact ($n = 36$ GLM, $F_{1,35} = 0.62$, $p = 0.007$), the interaction term for time to sample and type of restraint (physical or chemical) ($n = 36$ GLM, $F_{1,35} = 0.73$, $p = 0.01$) and vacutainer type ($n = 36$ GLM, $F_{1,35} = 28.8$, $p < 0.001$) (Table 4).

Table 3
Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in extracted plasma, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Time to obtain sample	0.28	0.18	0.14
Type of restraint	0.34	0.31	0.27
Vacutainer type	0.09	0.11	0.39
Interaction term between time to sample and type of restraint	−0.29	0.18	0.13

Table 4
Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in raw plasma, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Time to obtain sample	−0.53	0.18	0.007
Type of restraint (chemical)	−0.39	0.31	0.21
Vacutainer type (heparin)	−0.57	0.11	<0.001
Interaction term between time to sample and type of restraint	0.5	0.18	0.01

3.3. Correlations between oxytocin detected in raw and extracted plasma

No relationship was found between oxytocin concentrations detected in raw and extracted plasma samples taken with heparin ($n = 27$ GLM, $F_{1,26} = 1.24$, $p = 0.25$) or EDTA vacutainers ($n = 25$ GLM, $F_{1,24} = 2.47$, $p = 0.12$) (Fig. 1). Furthermore, when the best model was used to generate predicted oxytocin concentrations in extracted samples from a subset of raw values, the resulting dataset (mean = 2.1 ± 0.008 pg/ml) was significantly different from the true values (mean = 8.2 ± 0.4 pg/ml) when analysed using a Welch two sample *T* test ($t(15) = 8.2$, $p < 0.001$).

3.4. Clearance rate of oxytocin calculated using raw and extracted samples

Both GAM models showed that in raw and extracted plasma there was a significant decline in oxytocin over time towards the basal level ($n = 13$ in each GAM, for both $p < 0.001$). However the oxytocin values detected using raw plasma were much less successful at producing an accurate clearance curve compared to one generated with extracted plasma (percentage deviance explained: raw plasma: 30.7%, extracted plasma: 85.2%). In addition the confidence intervals were much wider around the fitted curve for the

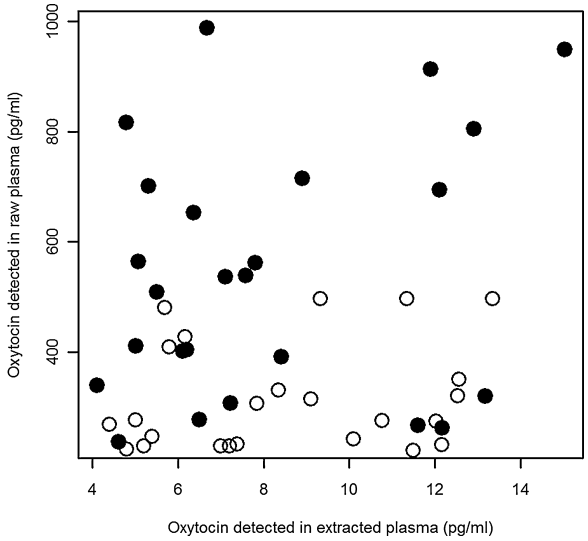


Fig. 1. Scatter plot showing the oxytocin concentrations detected in plasma samples that were analysed both raw and with extraction prior to assay. Samples collected in EDTA vacutainers are shown in black (●) and samples collected in heparin vacutainers are shown in white (○).

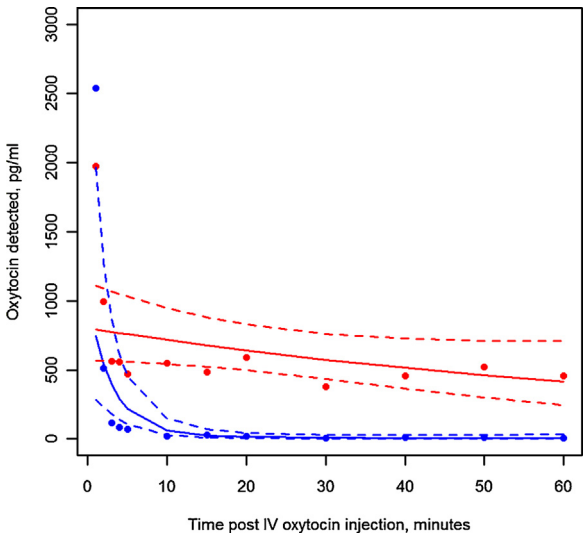


Fig. 2. Clearance curves for one individual given a single IV oxytocin dose, generated using both raw (red, the top solid line) and extracted (blue, the bottom solid line) plasma, with confidence intervals (dashed). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

raw results (Fig. 2). The plasma clearance rate calculated using the curve generated with extracted plasma was 0.035 L/min/kg, while the plasma clearance rate calculated using the curve generated with raw plasma was 0.004 L/min/kg.

4. Discussion

This study has determined successfully for the first time plasma oxytocin concentrations in two novel mammalian species, the grey seal and harbour seal. Furthermore, it has demonstrated clear and substantial differences in the oxytocin concentrations measured in phocid plasma samples depending on the protocol used to collect and analyse them. The largest differences were between raw and extracted plasma: raw samples produced oxytocin measures several hundreds times higher than extracted plasma. Importantly, no relationship was detected between the raw and extracted oxytocin concentrations. Such a relationship would be necessary for the prediction of one based on the other. Samples analysed raw were significantly influenced by the time it took to obtain a blood sample and by the type of restraints used, while extracted samples were not affected by these sources of variation. Finally samples taken with EDTA vacutainer tubes had significantly higher concentrations of oxytocin than heparin tubes when run raw on a plate, but when extracted there was no difference between the two. Therefore the design of a study protocol will have a major impact on the results generated, which must be taken into account when analysing and interpreting the data.

4.1. Raw versus extracted plasma

The results show clearly that the concentration of oxytocin detected in raw plasma samples is substantially higher than that in extracted samples (mean oxytocin extracted EDTA: 8.1 pg/ml, extracted heparin: 8.3 pg/ml, raw EDTA: 543.2 pg/ml, raw heparin: 300.9 pg/ml). These concentrations represent the first published data on plasma oxytocin concentrations found in a phocid seal, or any marine mammal species, and are comparable to both extracted and raw plasma concentrations reported in the literature for other mammalian species. Humans have been reported to have basal plasma oxytocin concentrations ranging between 0.1–23 pg/ml in extracted plasma and 99–405 pg/ml in raw plasma (reviewed in Szeto et al., 2011) and rats have mean oxytocin concentrations of 6.8 pg/ml in extracted plasma (Langraf, 1981) and concentrations ranging from 78.9–580 pg/ml in raw plasma (Carter et al., 2007; Martínez-Lorenzana et al., 2008). Szeto et al. (2011) detected the same contrast in concentrations measured using raw and extracted plasma while evaluating radioimmunoassay (RIA) and ELISA protocols for oxytocin analysis and used high performance liquid phase chromatography (HPLC) to investigate the components of raw plasma, enabling the identification of several oxytocin immunoreactive species in raw plasma. This accounts for the large difference between the concentrations of oxytocin measured in raw and extracted samples, and at least one of the additional immunoreactive species was identified as a degradation product of oxytocin. It is vital that any studies using raw plasma as the medium for analysis take this into account, as any results obtained will not be a reflection of oxytocin alone, but rather of an unknown number of other metabolic processes generating these reactive molecules in the blood.

The potential differences a study can incur in their results just by using raw or extracted plasma is well illustrated by the different plasma clearance rates generated from the clearance trial in this study. The plasma clearance rate for seals detected with extracted plasma (0.035 L/min/kg) is comparable to the existing values published for the clearance rate of oxytocin in other mammalian species detected with RIAs (0.027 L/kg/min in human men and 0.021 L/min/kg in human women (Leake et al., 1980), between 0.025 and 0.085 L/min/kg in rats depending on dose (Morin et al., 2008), and 0.016 L/min/kg in baboons (Kowalski et al., 1998)). However the plasma clearance rate generated by the raw plasma data (0.004 L/min/kg) is much slower than the rate from extracted samples and other published work due to the high AUC value generated from the raw plasma clearance curve (Fig. 2).

A difference between the two concentrations detected does not immediately rule out using information about one to infer the concentration of the other. If the other reactive species measured in the ELISA in raw samples are products of oxytocin metabolism then it could be theorised that raw levels would still give an indication of an individual's oxytocin activity in the blood. There have been studies that detected such a correlation, such as Michopoulos et al. (2011) and others that found no such link, such as Szeto et al. (2011). The current study was not able to find any correlation between the raw and extracted samples, and in addition found that regression models built on such data had poor predictive power for generating accurate extracted oxytocin levels from raw measurements. When analysing a set of serial samples taken during a clearance trial, the data generated from extracted samples produced a curve which accurately represented the decay rate. The data generated from raw samples, while still detecting an overall fall in oxytocin levels, could not have a clearance curve fitted to it that accounted for more than 30% of the variation in the data. Therefore our findings are in agreement with Szeto et al.'s work, that raw plasma cannot be used to accurately analyse oxytocin levels. However, Michopoulos et al. used serum rather than plasma for their analysis, which may have produced differences in the metabolites maintained or denatured during collection and storage. In addition the datasets used here and by Szeto et al. to generate the regressions and correlations are larger ($n = 52$ and $n = 39$, respectively) than the one used by Michopoulos et al. ($n = 11$) and therefore may have obtained a more representative dataset to work from. Michopoulos et al. also do not give details of the correlation model used to generate their results, preventing direct comparisons between the varying methods of statistical analysis used in the current study (GLM) and by Szeto et al. (Spearman rank correlation).

Therefore we caution that studies using raw plasma with the Assay Designs ELISA kit are unlikely to report oxytocin alone and additionally cannot accurately infer this data from their results.

4.2. Heparin versus EDTA vacutainers

The current study shows that if plasma is extracted prior to analysis, the particular anticoagulant in the vacutainer makes no difference to the concentration of oxytocin detected. This allows studies using extracted plasma to be comparable, regardless of vacutainer choice. However when analysing raw plasma, EDTA tubes contain almost double the concentration of oxytocin compared to heparin tubes collected samples (mean oxytocin EDTA: 543.2 pg/ml, heparin: 300.9 pg/ml). Other studies analysing different biological compounds in human samples have reported similar differences between the anticoagulants (Dong et al., 2010; Gonzalez-Covarrubias et al., 2013) while for some peptides there appears to be no difference (Varo et al., 2006). The protocol instructions for the Assay Designs ELISA do state that the chelators in EDTA might affect the activity of the conjugate used in the kit (Enzo Life Sciences Oxytocin ELISA kit Manual 2012). The conjugate is responsible for reacting with the *p*-nitrophenylphosphate substrate to generate a yellow colour, which indicates a low concentration of oxytocin as the intensity of the colour increases. If this function was impaired due to EDTA presence then no yellow colour would be generated and a false positive would occur. Heparin vacutainers work by potentiating the action of antithrombin (Chuang et al., 2001), which inactivates coagulation proteins rather than using chelators to bind ions, and this may account for the difference in results. This highlights the importance of fully investigating the consequences of any deviation from the kit manufacturer's instructions as the kit protocol recommends using EDTA tubes for collection with extracting prior to analysis. Therefore the discrepancy between the two tube types must be taken into account when designing a study, interpreting the results and comparing them across studies, and can be avoided completely if samples are extracted.

4.3. Influence of other variables

Several variables were analysed alongside protocol type to ensure all sources of variation in the dataset could be identified. The time to obtain a plasma sample and using physical or chemical restraints had no effect on the oxytocin detected in extracted samples, but significantly impacted on the concentrations detected in raw plasma. Oxytocin has been linked to restraint stress in rodents (Grippo et al., 2009) and is hypothesised to rise in response to high cortisol levels. However in previous studies only long term capture and restraint generated peaks in oxytocin (CS Carter, personal communication) and our results support this. As there were no significant changes in oxytocin concentration with time in extracted samples, it should be possible to obtain samples in future studies that allow detection of basal oxytocin concentrations from wild animals captured either manually or using a chemical immobiliser providing samples are extracted prior to analysis.

The individual's species and sex were both significant sources of variation in this study, while time of year was not. As this work includes few individuals, and no repeats of any species/sex combination were possible, stochastic differences between them are entirely plausible, and having access to a larger number of animals would have allowed us to examine this variation to determine its validity. Despite, there being significant variation in individual's oxytocin concentration, the differences generated by the four protocols are greater still and are present at the highest level of significance in the model.

Finally this study focused on the variation in oxytocin generated by extraction protocol and vacutainer choice, and did not specifically test for variation generated by differing storage temperatures or the use of aprotinin to stabilise the peptide. These two variables were kept consistent across the samples in this study, with all plasma stored at −20 °C with no addition of aprotinin. The coefficient of variance in this study remained consistently below 5% across two years of analysis, indicating that degradation of oxytocin in samples stored at −20 °C without the use of aprotinin was negligible. The influence of storage temperature and additional inhibitors on oxytocin concentration detected would be an area worthy of future study, as protocols in the literature vary on these points along with vacutainer type and use of extraction. The majority of studies published freeze at the recommended −70 °C with a minority using −20 °C or below (Scantamburlo et al., 2007; Yayou et al., 2010), yet this study and several others (Szeto et al., 2011; van der Post et al., 1997) indicate that oxytocin may be more stable in warmer storage temperatures than is thought currently. The use of aprotinin or any inhibitor post-sampling is extremely diverse in the literature, with as many studies using aprotinin (Feldman et al., 2010a,b; Skrundz et al., 2011; Weisman et al., 2013; Hoffman et al., 2012) as not (Scantamburlo et al., 2007; Yayou et al., 2010; Szeto et al., 2011; Deisenhammer et al., 2012; Hoge et al., 2012). The impact of these additional inconsistencies between studies in oxytocin analysis protocol would benefit from further investigation.

In conclusion, the protocol followed when collecting and analysing plasma for oxytocin does have a significant impact on the results generated and experimental design should be carefully considered when planning research in this area. The concentrations of basal plasma oxytocin in phocid seals are consistent with those detected in all mammal species studied to date in both extracted and raw plasma, and the clearance rate for this hormone in phocids is also comparable to that found in humans (Leake et al., 1980), rats (Morin et al., 2008) and primates (Kowalski et al., 1998). Therefore despite being novel species to this field, it appears that phocid seals represent a typical mammalian system in terms of plasma oxytocin. Therefore future work should use methods which generate reliable, repeatable measures of plasma oxytocin, as well as developing protocols for using other peripheral substrates such as saliva and urine. Due to their non-invasive nature, such mediums are becoming more popular but the methodologies to utilise them have their critics (Horvat-Gordon et al., 2005). Without a firm knowledge of what experimental variables researchers may inadvertently introduce into a study, it will be extremely difficult to decipher the role of this neuropeptide in human and animal social and reproductive systems.

Acknowledgements

We would like to thank Simon Moss, Ryan Milne, Alicia Widmer and everyone that assisted in caring for the seals during their time in captivity and in handling them during sampling events. We would also like to thank Ailsa Hall for her valuable advice on ELISA protocol and troubleshooting.

This work was conducted as part of a PhD, which was funded by the Natural Environment Research Council (NERC), UK and by SMRU Marine, St Andrews, UK. The funding sources had no role in the planning, execution, analysis or writing of this study.

References

Amico JA, Challinor SM, Cameron JL. Pattern of oxytocin concentrations in the plasma and cerebrospinal fluid of lactating rhesus monkeys (*Macaca mulatto*): evidence for functionally independent oxytocinergic pathways in primates. J Clin Endocrinol Metab 1990;71:1531–5.

Bales KL, van Westerhuyzen JA, Lewis-Reese AD, Grotte ND, Lanter JA, Carter CS. Oxytocin has dose dependant developmental effects on pair-bonding and alloparental care in female prairie voles. Horm Behav 2007;52:274–9.

Baskerville TA, Douglas AJ. Dopamine and oxytocin interaction underlying behaviours: potential contributions to behavioural disorders. CNS Neurosci Ther 2010;16:e92–123.

Carter CS, Pournajafi-Nazarloo H, Kramer KM, Ziegler TE, White-Traut R, Bello D, et al. Oxytocin: behavioural associations and potential as a salivary biomarker. Ann N Y Acad Sci 2007;1098::312–22.
Chuang YJ, Swanson R, Srikumar M, Raja M, Olson ST. Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. J Biol Chem 2001;276:14961–71.
Crockford C, Wittig RM, Langergraber K, Ziegler TE, Zuberbühler K, Deschner T. Urinary oxytocin and social bonding in related and unrelated wild chimpanzees. Proc R Soc Lond B Biol Sci 2013;280:20122765.
Cool DR, DeBrosse D. Extraction of oxytocin and arginine–vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption–ionization time-of-flight mass spectrometry. J Chromatogr B 2003;792:375–80.
Dale HH. On some physiological actions of ergot. J Physiol (Lond) 1906;34:163–206.
Deisenhammer EA, Hofer S, Scwitzer O, Defrancesco M, Lemmler G, Wildt L, et al. Oxytocin plasma levels in psychiatric patients with and without recent suicide attempt. Psychiatry Res 2012;200:59–62.
Devarajan K, Rusak B. Oxytocin levels in the plasma and cerebrospinal fluid of male rats: effects of circadian phase, light and stress. Neurosci Lett 2004;367:144–7.
Dong N, Dong J, Liu P, Xu L, Shi S, Wu Q. Effects of anticoagulants on human plasma soluble corin levels measured by ELISA. Clin Chim Acta 2010;14:1998–2003.
El-Masry N, Soliman A, Moety HA. Alterations of prolylendopeptidase, oxytocin and vasopressin activity in the plasma of autistic children. Curr Psychiatry 2010;17:31–7.
Feldman R, Gordon I, Schneiderman I, Weisman O, Zagoory-Sharon O. Natural variations in maternal and paternal care are associated with systematic changes in oxytocin following parent–infant contact. Psychoneuroendocrinology 2010a;35:1133–41.
Feldman R, Gordon I, Zagoory-Sharon O. The cross-generation transmission of oxytocin in humans. Horm Behav 2010b;58:669–76.
Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function and regulation. Physiol Rev 2001;81:629–83.
Gonzalez-Covarrubias V, Dane A, Hankemeier T, Vreeken RJ. The influence of citrate, EDTA and heparin anticoagulants to human plasma LC-MS lipidomic profiling. Metabolomics 2013;9:337–48.
Grippo AJ, Trhanas DM, Zimmerman RR, Porges SW, Carter CS. Oxytocin protects against negative behavioural and autonomic consequences of long term social isolation. Psychoneuroendocrinology 2009;34:1542–53.
Haaland P, Samarov D, McVey E. calibFit: statistical models and tools for assay calibration. R package version 2.1.0/r17; 2011 http://R-Forge.R-project.org/projects/calibfun/
Hall AJ. Blood chemistry and hematology of grey seal (*Halichoerus grypus*) pups from birth to postweaning. J Zoo Wildl Med 1998;29:401–7.
Heinrichs M, Baumgartner T, Kirschbaum C, Ehlert U. Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. Soc Biol Psychiatry 2003;54:1389–98.
Hoffman ER, Brownley KA, Hamer RM, Bulik CM. Plasma, salivary and urinary oxytocin in anorexia nervosa: a pilot study. Eat Behav 2012;13:256–9.
Hoge EA, Lawson EA, Metcalf CA, Keshaviah A, Zak PJ, Pollack MH, et al. Plasma oxytocin immunoreactive products and response to trust in patients with social anxiety disorder. Depress Anxiety 2012;29:924–30.
Hollander E, Novotny S, Hanratty M, Yaffe R, DeCaria CM, Aronowitz BR, et al. Oxytocin infusion reduces repetitive behaviours in adults with autistic and Asperger's disorders. Neuropsychopharmacology 2003;28:193–8.
Holt-Lunstad J, Birmingham W, Light KC. The influence of depressive symptomatology and perceived stress on plasma and salivary oxytocin before, during and after a support enhancement intervention. Psychoneuroendocrinology 2011;36:1249–56.
Horvat-Gordon M, Granger DA, Schwartz EB, Nelson VJ, Kivlighan KT. Oxytocin is not a valid biomarker when measured in saliva by immunoassay. Physiol Behav 2005;84:445–8.
Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E. Oxytocin increases trust in humans. Nature 2005;435:673–6.
Kowalski WB, Diveky L, Mehendale R, Parsons M, Wilson L Jr. Effect of pregnancy on the metabolic clearance rate and the volume of distribution of oxytocin in the baboon. Am J Physiol Endocrinol Metab 1998;274:791–5.
Kramer KM, Cushing BS, Carter CS, Wu J, Ottinger MA. Sex and species differences in plasma oxytocin using an enzyme immunoassay. Can J Zool 2004;82:1194–200.
Langraf R. Simultaneous measurement of arginine vasopressin and oxytocin in plasma and neurohypophyses by radioimmunoassay. Endokrinologie 1981;78:191–204.
Leake RD, Weitzman RE, Fisher DA. Pharmokinetics of oxytocin in the human subject. Obstet Gynecol 1980;56:701–4.
Leake RD, Weitzman RE, Fisher DA. Oxytocin concentrations during the neonatal period. Neonatology 1981;39:127–31.
Levine A, Zagoory-Sharon O, Feldman R, Weller A. Oxytocin during pregnancy and early postpartum: individual patterns and maternal–fetal attachment. Peptides 2007;28:1162–9.
Lévy F, Kendrick KM, Keverne EB, Piketty V, Poindron P. Intracerebral oxytocin is important for the onset of maternal behaviour in inexperienced ewes delivered under peridural anesthesia. Behav Neurosci 1995;106:427–32.
MacDonald K, MacDonald TM. The peptide that binds: a systemic review of oxytocin and its prosocial effects in humans. Harv Rev Psychiatry 2010;18:1–21.
Maestripieri D, Hoffman CL, Anderson GM, Carter CS, Higley JD. Mother–infant interactions in free-ranging rhesus macaques: relationships between physiological and behavioural variables. Physiol Behav 2009;96:613–9.
Martínez-Lorenzana G, Espinosa-López L, Carranza M, Aramburo C, Paz-Tres C, Rojas-Piloni G, et al. PVN electrical stimulation prolongs withdrawal latencies and releases oxytocin in cerebrospinal fluid, plasma and spinal cord tissue in intact and neuropathic rats. Pain 2008;140:265–73.
McCarthy MM. Oxytocin inhibits infanticide in female house mice (*Mus domesticus*). Horm Behav 1990;24:365–75.
McCullough ME, Churchland PS, Mendez AJ. Problems with measuring peripheral oxytocin: can the data on oxytocin and human behaviour be trusted? Neurosci Biobehav Rev 2013;37:1485–92.
Michopoulos V, Checchi M, Sharpe D, Wilson ME. Estradiol effects on behaviour and serum oxytocin are modified by social status and polymorphisms in the serotonin transporter gene in female rhesus monkeys. Horm Behav 2011;59:528–35.
Modahl C, Green L, Fein D, Morris M, Waterhouse L, Feinstein C, et al. Plasma oxytocin levels in autistic children. Soc Biol Psychiatry 1998;43:270–7.
Morin V, del Castillo JRE, Authier S, Ybarra N, Otis C, Gauvin D, et al. Evidence for nonlinear pharmacokinetics of oxytocin in anesthetized rat. J Pharm Pharmaceut Sci 2008;11:12–24.
Moscovice LR, Ziegler TE. Peripheral oxytocin in female baboons relates to estrous state and maintenance of sexual consortships. Horm Behav 2012;62:592–7.
Neumann ID, Landgraf R. Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviours. Trends Neurosci 2012;35:649–59.
Nagasawa M, Kikusui T, Onaka T, Ohta M. Dog's gaze at its owner's urinary oxytocin during social interaction. Horm Behav 2009;56:434–41.
Neumann ID. Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. J Neuroendocrinol 2008;20:858–65.
Odendaal JS, Meintjes RA. Neurophysiological correlates of affiliative behaviour between humans and dogs. Vet J 2003;165:296–301.
Popik P, Vetulani J, Van Ree JM. Low doses of oxytocin facilitate social recognition in rats. Psychopharmacology 1992;106:71–4.
Prakash BS, Vijay P, Kliem H, Kulozik U, Meyer HHD. Determination of oxytocin in milk of cows administered oxytocin. Anal Chim Acta 2009;636:111–5.
R Development Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistics Computing, Vienna, Austria. 2008 http://www.R-project.org
Ross HE, Young LJ. Oxytocin and the neural mechanisms regulating social cognition and affiliative behaviour. Front Neuroendocrinol 2009;30:534–47.
Scantamburlo G, Hansenne M, Fuchs S, Pitchot W, Marechal P, Pequeux C, et al. Plasma oxytocin levels and anxiety in patients with major depression. Psychoneuroendocrinology 2007;32:407–10.
Skrundz M, Bolten M, Nast I, Hellhammer DH, Meinlschmidt G. Plasma oxytocin concentration during pregnancy is associated with development of postpartum depression. Neuropsychopharmacology 2011;36:1886–93.
Szeto A, McCabe PM, Nation DA, Tabek BA, Rossetti MA, McCullough ME, et al. Evaluation of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin. Psychosom Med 2011;73:393–400.
Uvnäs-Moberg K. Oxytocin may mediate the benefits of positive social interaction and emotions. Psychoneuroendocrinology 1998;23:819–35.
van der Post JAM, van Buul BJA, Hart AAM, van Heerikhuize JJ, Pesman G, Legros JJ, et al. Vasopressin and oxytocin levels during normal pregnancy: effects of chronic dietary sodium restriction. J Endocrinol 1997;152:345–54.
Varo N, Nuzzo R, Natal C, Libby P, Schönbeck U. Influence of pre-analytical and analytical factors on soluble CD40L measurements. Clin Sci 2006;111:341–7.
Weisman O, Zagoory-Sharon O, Schneiderman I, Gordon I, Feldman R. Plasma oxytocin distributions in a large cohort of women and men and their gender-specific associations with anxiety. Psychoneuroendocrinology 2013;38:694–701.
Wood S. Generalized additive models: an introduction with R. Florida, USA: Chapman and Hall/CRC; 2006a.
Wood S. Low-rank scale-invariant tensor product smooths for generalized additive mixed models. Biometrics 2006b;62:1025–36.
Wood S. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J R Stat Soc B 2011;73:3–36.
Wood S. http://cran.r-project.org/web/packages/mgcv/index.html
Yayou K, Ito S, Yamamoto N, Kitagawa S, Okamura H. Relationship of stress responses with plasma oxytocin and prolactin in heifer calves. Physiol Behav 2010;99:362–9.