

The role of the Vomeronasal Organ in the endocrine response of does in oestrus

By

J. Jansen van Vuuren

Submitted to the Department of Animal and Wildlife Sciences

Faculty of Natural and Agricultural Sciences

University of Pretoria

For the requirements of the degree MSc (Agric) Animal Production Management

Pretoria

April 2015

Contents

Acknowledgements	4
List of Abbreviations	5
List of Tables and Graphs	7
Abstract	8
Chapter 1: Literature review	10
1.1 Introduction	10
1.2 Anatomy of the Vomeronasal Organ	12
1.3 The Vomeronasal-Hypothalamic Axis	14
1.3.1 Neural Connections of the Vomeronasal Organ, the Accessory Olfactory Bulb and the Hypothalamus	14
1.3.2 The Physiological Relationship between the Vomeronasal System and the hypothalamus	15
1.4 Pheromone Reception by the Vomeronasal Organ	18
1.4.1 Pheromones and the Vomeronasal System	18
1.4.1.1 Vomeronasal Receptor Function	22
1.4.2 The Vomeronasal Organ and Flehman	22
1.5 Outline and Aim of this study	25

Chapter 2: Materials and Methods	26
2.1 Animals	26
2.2 Treatment	26
2.3 Feed	29
2.4 Medication	29
2.5 Blood Collection	30
2.6 Statistical analysis	32
Chapter 3: Results and Discussion	33
3.1 Results	33
3.2 Discussion	39
Conclusion	46
Bibliography	48

Acknowledgements

This study would not have been possible without the help and guidance of Professor E.C. Webb from the Department of Animal and Wildlife Sciences, who suggested that I do this research and dissertation, and to Professor K.K. Booth from the Department of Anatomy and Physiology, Faculty of Veterinary Sciences, for his willingness to be a co-supervisor for this degree. Both of these departments are at the University of Pretoria, South Africa.

This thesis was also made possible by my employer, the National Wool Growers' Association of South Africa (NWGA), who funded part of this study.

I am forever thankful to:

My parents, who supported me throughout my under-graduate degree and for being tough on me from a young age so that I would be able to be successful later in life.

My wife Lyndal, and our son Xavier, for your understanding and support at difficult times while I was writing this dissertation. I love you very much.

List of Abbreviations

1. AOB: Accessory Olfactory Bulb
2. AOS: Accessory Olfactory System
3. BnST: Bednucleus of the Stria Terminalis
4. cAMP: Cyclic Adenyl Monophosphate
5. CRH: Corticotropin Releasing Factor
6. DBH: Dopamine- β -hydroxylase
7. FSH: Follicle Stimulating Hormone
8. FSH-RF: Follicle Stimulating Hormone Releasing Factor
9. GC: Guanylate Cyclase
10. GnRH: Gonadotropin Releasing Hormone
11. IL-1: Interleuken 1
12. IL-1 α : Interleuken 1 α
13. LH: Luteinizing Hormone
14. LHRH: Luteinizing Hormone Releasing Hormone
15. MNA: Medial Nucleus of the Amygdala
16. MOB: Main Olfactory Bulb
17. MOS: Main Olfactory System
18. NAOT: Nucleus of the Accessory Olfactory Tract
19. NE: Norepinephrine
20. NO: Nitric Oxide
21. NvS: Non-volatile Stimuli
22. OCAM: Olfactory Cell Adhesion Factor
23. OR: Olfactory Receptor
24. P: Significance Level
25. PCNA: Posterolateral Cortical Nucleus of the Amygdala
26. PGE₂: Prostaglandin E₂
27. PGF_{2 α} : Prostaglandin F_{2 α}
28. PIF: Prolactin Inhibiting Factor
29. PLA2: Phospholypase 2

- 30. PMCNA: Posteromedial Cortical Nucleus of the Amygdala
- 31. PMSG: Pregnant Mare Serum Gonadotropin
- 32. POA: Preoptic Area
- 33. PRF: Prolactin Releasing Factor
- 34. VNO: Vomeronasal Organ
- 35. VMNH: Ventromedial Nucleus of the Hypothalamus
- 36. VR: Vomeronasal Receptor

List of Tables and Graphs

Table 2.1 Oestrus synchronization of does in VNO trail.

Table 2.2 Demonstrating day-by-day activities of the trial.

Table 3.1 Average values (\pm SD) of estradiol, LH and cortisol of both groups (Control (C) and Treatment (T)) during the different period intervals (T_1 - T_9).

Table 3.2 Significance of the difference between the average values of the treatment and control groups are given for estradiol, LH and cortisol.

Graph 3.1 Average estradiol concentrations in control (Est_c) and treatment (Est_T) groups for the trial period.

Graph 3.2 Average estradiol concentration in control and treatment groups displayed over time, with standard deviations also indicated for each group ($T_1 - T_9$).

Graph 3.3 Average LH concentration in control (LH_c) and treatment (LH_T) groups for the entire trial period.

Graph 3.4 Average plasma LH concentration in control and treatment groups displayed over time, with standard deviations also indicated for each group ($T_1 - T_9$).

Graph 3.5 Average cortisol in control ($Cort_c$) and treatment ($Cort_T$) groups for the trial period.

Graph 3.6 Average $Cort_c$ and $Cort_T$ displayed over time, with standard deviations also indicated for each group ($T_1 - T_9$).

The role of the Vomeronasal Organ in the endocrine response of does in oestrus

By

Jacques Jansen van Vuuren

Promotor: Prof. E.C. Webb
Co-supervisor: Prof. K.K. Booth
Faculty: Faculty of Natural and Agricultural sciences
Department: Department of Animal and Wildlife sciences
Degree: MSc (Agric) Animal Production Management

Abstract

The seasonality of breeding activity in sheep and goats represents an important restraint in the breeding program of commercial flocks. The “Whitten effect” or “male effect” is one of the techniques available to make this restraint less prominent. The Vomeronasal organ, in females, is involved in receiving pheromones produced by the male animal so that the female’s reproductive cycle can change from one being in anoestrus to one in cycling oestrus. Various hormones are involved in the reproductive cycle of female animals, for instance estradiol, luteinizing hormone (LH), cortisol, luteinizing hormone releasing hormone, amongst others. Studies have indicated a direct neural connection from the sensory cells in the VNO to the cells responsible for the production and secretion of reproductive endocrine hormones. The H_0 hypothesis is that the Vomeronasal organ has no effect on the secretion of endocrine hormones and that it plays no part in the reproduction process of small stock, particularly in goats.

Twenty-one multiparous does were used, 11 in the treatment group and 10 in the control group. Treatment consisted of cauterization of the Vomeronasal organ, while the control group had their nasal cavity irrigated with physiological saline under anaesthesia. All of the animals were synchronized by way of vaginal sponges and 0.5ml Estrumate® injection per doe on day one of the trial. The does were rested for 21 days after synchrony before the rams were introduced. Rams and does were kept together for 5 days, from days 32 to 36 of the trial. Pregnancy scans were done 40 days after the rams were taken away from the does. Hormone levels were

measured by means of the Beckman Coulter Access Immunoassay System (a paramagnetic particle, chemiluminescent immunoassay).

Over the entire trial period, average estradiol values were 111.85 pmol/L for the control group and 105.82 pmol/L for the treatment group. Average LH values for the control group were 0.31 IU/L and 0.23 IU/L for the treatment group. Cortisol average values were 29.12 nmol/L for the control group and 42.14 nmol/L for the treatment group. Means differed for estradiol between the two groups were significant at T₄, T₅, T₆, T₈ and T₉, for LH at T₄, T₅, T₆ and T₈ and for cortisol at T₂, T₆, T₇ and at T₉. Over all means between the control and treatment group only differed significantly for cortisol.

The results of this study shows that the introduction of the bucks influences the secretion of reproduction hormones. Estradiol concentrations were near normal with no significant differences between the two groups. This indicates that ovarian function was normal in both groups. Luteinizing hormone is the most important hormone which is secreted for the initiation of the reproduction process. In the treatment group there was a marginal difference in the overall mean concentration of LH compared to the control group. A statistically significant absence of the primary LH peak that is required for ovulation to occur was observed in the treatment group. A cascade of hormones needs to be activated and secreted for does to show sexual activity, go into oestrus and accept the male animal. This was illustrated in the control group where the signals, secretion of hormones and sexual activity were normal. During the introduction of the males, an increase in cortisol levels in the treatment group, with a non-functional Vomeronasal organ, indicated increase in stress levels of these animals due to their inability to recognise the other animals introduced into the group, even though this group had use of their main olfactory organ. All of the does in the control group became pregnant but none of the treatment group fell pregnant. The results of this study thus indicate that the VNO plays a critical role in reproduction, animal recognition and flock management in goats.

Keywords: Vomeronasal organ, luteinizing hormone, pheromones, oestrus, goats.

Chapter 1: Literature review

1.1 Introduction

Annual photoperiod variation is the most widespread proximate factor timing seasonal reproduction. Photoperiod is a very reliable predictor of future environmental conditions, since it is a very constant environmental cue (Bearden et al, 2004). Some species (i.e., sheep, goat, and deer) are known as 'short-day breeders' because their breeding season occurs mainly when days are getting shorter (autumn). Short-day breeding species normally have gestation lengths of 5-6 months and their offspring are usually born in spring (Bearden et al, 2004).

When females are grouped together, a certain degree of oestrus synchronization occurs (Stabenfeldt and Edqvist, 1993). The oestrus cycle is the period of time from the beginning of one heat period to the start of the next. In sheep the average oestrus length is 17 days and in goats it is 21 days. Oestrus can last for 24-36 hours in ewes and 30-40 hours in does (Bearden et al, 2004). A doe in oestrus is restless, bleats and urinates frequently. She may even wag her tail rapidly, experience a loss in appetite and rub against other does. Her vulva might become red, swollen and there might even be a thin mucous discharge (Faruk et al, 2004).

Interactions between sexes

Different sexes of the same breed influence one another in some way or another, especially male-to-female interactions. This phenomenon has been described for a variety of mammalian species including mice, rats, sheep and goats (Rosa and Bryant, 2002). When these females are introduced to a male, certain physiological and even physical changes can be observed (Rosa and Bryant, 2002; Over et al, 1990). These include:

1. The Whitten effect - rapid synchronization of oestrus in previously unsynchronized females (McClintock, 1978; Chemineau, 1983; Martin et al, 2004).
2. The Bruce effect - the blockage of pregnancy (Bruce, 1960).
3. The acceleration of puberty in young individuals of the opposite gender (Vandenburgh, 1967, 1971).
4. Testicular development in males (Whitsett et al, 1984).
5. An increase in reproductive function outside the natural breeding season (Martin et al, 1986; Knights et al, 2002; Avdi et al, 2004; Ungerfeld et al, 2003).

The effect that rams have on ewes is well documented, but there are more interactions than male-to-female interactions. Rams also get affected by ewes. Ungerfeld (2003) showed that rams begin to show sexual behaviour before ewes when put together with ewes in oestrus and this is due to the fact that the male

reproductive system is activated before the female reproductive system. This activation includes an increase in the pulsatility of luteinizing hormone (LH) and increasing concentrations of testosterone and follicle-stimulating hormone (FSH) (Ungerfeld, 2003). According to Rosa and Bryant (2002) there are more interactions to take note of, for example: (1) Male-to-male interaction where males are reared far away from ewes, which facilitate the formation of social relationships among males and this may delay or even prevent the development of sexual interest in females. (2) Female-to-female interaction can occur when the sudden introduction of oestrus ewes is capable of inducing and synchronizing ovulation to seasonally anoestrus ewes and due to this interaction advancing the onset of the breeding season.

Although these observations have been well documented, the mechanisms involved are still somewhat unclear. In rodents the accessory olfactory system is primarily involved (McClintock, 1978; Reynolds and Keverne, 1979, Chemineau, 1983; Wysocki et al, 1985; Martin et al, 2004,). Some investigations suggest that the main olfactory system, situated in the nasal cavity, is primarily involved in ungulates' reproductive processes (Cohen-Tannoudji et al, 1989). Their main focus was on the neuro-anatomical connections between the main olfactory system and the hypothalamus, and they used various methods to disable the accessory olfactory system or its projections. These techniques involved the physical destruction of the Vomeronasal organ (VNO) itself, the severing of its neural projections (Cohen-Tannoudji et al, 1989), and anaesthetizing certain parts of the vomeronasal pathway. These studies found that a blockade of the vomeronasal neural projections had no effect on the LH response of anoestrus ewes when they were exposed to male odours.

The first aspect to consider in these investigations done by Cohen-Tannoudjie et al (1989), is the close anatomical position of the vomeronasal and olfactory neural pathways. When physically destroying or anaesthetizing the neural pathways of either system it is very possible to damage the other (Reynolds and Keverne, 1979), with the risk of attributing observations to the wrong system. There is also evidence of an alternative pathway from the accessory olfactory bulb (AOB) to the hypothalamus (Raisman, 1972). Sexual experience has been shown to affect the response of individuals to certain treatments. Fos expression was measured in both the vomeronasal and main olfactory pathways in sexually experienced and naïve male hamsters (Raisman, 1972). Firstly, it was found that fos expression in the medial preoptic area was more effectively stimulated by chemosensory stimulation in experienced males than in naïve males. Secondly, it was found that removal of the Vomeronasal organ had no effect on fos expression in experienced males, but severely affected naïve males. These naïve males expressed severely impaired mating function (Fewell and Meredith, 2002; Keverne, 2004). Reynolds and Keverne (1979) found that it is possible to suppress oestrus in sexually naïve female mice by grouping them together. In further investigations the Vomeronasal organs of

some grouped females were surgically removed and these females were cyclic 14 days after surgery, despite still being grouped (Reynolds and Keverne, 1979).

Biochemical mechanism of interaction between sexes

There is also evidence of a biochemical link between the final termination site of the vomeronasal neural projections and the hypothalamic control of the secretion of GnRH. This includes substances like norepinephrine (NE) and nitric oxide (NO) (Clarke et al, 2005). Some evidence suggests that the opioidergic system is also involved, especially β -endorphin (Bouret et al, 2000).

1.2 Anatomy of the Vomeronasal Organ

Pheromones detected by the VNO are known to influence a variety of animal activities, from sexual maturation to maternal offspring recognition during nursing (Johns et al, 1978). Receptors detecting pheromones have only been identified in the VNO and not in the nasal cavity (Dulac and Axel, 1995). Bailey (1977) indicated that flehman can be a possible mechanism by which stimuli can be delivered to the VNO.

Studies done by Abass et al (2012) on Iraqi sheep (Alawasi) showed that the VNO is located in the anterior portion of the nasal septum's floor (ventrolaterally). They also found that it has a tubular crescent shape, 6cm in length and opens cranially in the incisive duct. In these sheep, the VNO's caudal blind endings is found at the level of the second upper premolar tooth. The Vomeronasal organ consists of two, blind ending tubes, situated on either side of the base of the nasal septum, in the rostral part of the nasal cavity. These tubes are connected to the nasopalatine ducts, which connect the oral and nasal cavities. The organ is encapsulated by a C-shaped cartilage. The lumen is also C-shaped and lies to the medial side of a thickened area of connective tissue. This area of connective tissue contains various mucous glands and blood vessels. Studies done by Keller et al (2012) also indicate that the VNO in most mammals is located in the base of the nasal septum, as stated above.

The VNO contains two very distinct types of epithelium: sensory olfactory epithelium and ciliated respiratory epithelium (Dellmann, 1976). Sensory epithelium predominates in the posterior and medial parts of the lumen. Electron micrograph studies showed that the sensory epithelium is made up of three types of cells: basal cells, supporting cells and sensory cells (Dellmann, 1976). Clusters of microvilli that extend from the sensory cells characterize the surface of the sensory olfactory epithelium (Dellmann, 1976). Each cluster arises from one sensory cell and these clusters are separated by areas with relatively few microvilli. Within each cluster, there is a single microprocess (a larger projection), two to three times the dimensions of the microvilli. The axonal extensions of the sensory cells form the vomeronasal nerve fibres which leave the epithelium either as a single

fibre or in bundles. From their origin, these bundles are distinctly non-myelinated, however at the level of the lamina propria they form larger bundles of 15-20 axons which are myelinated (Dellmann, 1976). The supporting cells are long and narrow and the apical surface is mostly smooth and separates the sensory clusters from each other. The basal cells have a characteristic pyramidal shape, and possess numerous cytoplasmic processes that form a complex system of junctions with other basal cells and supporting cells (Dellmann, 1976). In certain areas, in between the sensory epithelium, a glandular crypt replaces the sensory epithelium. These crypts consist of mucus secreting cells with ciliated, columnar cells and they share the same basal cells as the sensory epithelium (Dellmann, 1976).

The rest of the lumen, the lateral and posterior parts, contains non-sensory, glandular epithelium and consists predominately of ciliated cells. Interspersed between these ciliated cells, cells resembling mucus-secreting (goblet) cells are found. The surface of these non-sensory cells has a characteristic ciliated edge, interspersed with fringes of microvilli. Glandular tissue, surrounded by collagenous tissue, is found in the lamina propria below this. These glands receive nerve supply from both myelinated and non-myelinated fibres and the secretory cells that make up the glandular tissue are essentially mucus secreting in nature (Kappers et al, 1936; Dellmann, 1976; Keller et al, 2012).

Takami (2002) describes five types of VNO's, based on the characteristics of the sensory epithelium, ranging from complex to simple.

- Type one is mostly found in snakes and is considered to be the most complex where the VNO is almost spherical in shape. The non-sensory epithelium is situated on a mushroom-like bulge opposite the sensory epithelium, with the lumen separating the epithelial layers. The sensory epithelium consists of a superficial layer of supporting cells and an underlying layer of sensory cells. The sensory cell layer is divided by connective tissue septi into small columns. Blood vessels are restricted to the septi. Each column contains 10-30 sensory neurons.
- Type two is predominant in rodents. In these species the connective tissue penetrates into the sensory epithelium, thus blood vessels are frequently found within the epithelial tissues.
- Type three is found in marsupials (opossum), ungulates (horse, bovine, ovine), carnivores (canine, feline, and ferret), and prosimian and platyrrhine primates are classified as type three VNO which are very similar to type two, but intra-epithelial blood vessels have not been described thus far in type three VNO's (Halpern and Martinez-Marcos, 2003).
- Type four VNO is mostly found in amphibians and is not situated in a separate tube. The lumen forms part of the nasal cavity and non-sensory epithelium is absent.

- The fifth type of VNO is found in the human foetus and appears to be rudimentary after birth. Its function remains unknown. Chimpanzees also have this type of VNO.

1.3 The Vomeronasal-Hypothalamic Axis

1.3.1 Neural Connections of the Vomeronasal Organ, the Accessory Olfactory Bulb and the Hypothalamus.

Early in the twentieth century various investigators found evidence for the existence of two olfactory systems in most species of mammals (Kappers et al, 1936; Lohman and Mentink, 1969). It was found that most mammals, apart from the higher primates, have two types of olfactory receptors. These are the neurosensory receptors of the main olfactory system, which resides in the olfactory mucosa of the nasal cavity, and the specialized receptors of the accessory olfactory system which is found in the Vomeronasal organ (Kappers et al, 1936). Kappers et al (1936) found that these receptors projected fibres to different centers in the brain. The VNO receptors projected fibres to the accessory olfactory bulb (AOB), while the nasal olfactory receptors projected fibres to the main olfactory bulb (MOB). It was found that fibres from the main olfactory bulb pass through the accessory olfactory bulb and this made attempts to trace the nerve fibres from the two olfactory bulbs to their termination points very complicated (Lohman and Mentink, 1969). Thus, lesions of the AOB fibres would inevitably include fibres from the MOB (Lohman and Mentink, 1969). These investigations did, however, present some evidence for the existence of a dual olfactory system in vertebrates. Raisman (1972) traced the cortical nerve projections of both the AOB and the MOB. His findings further demonstrated the existence of two separate olfactory systems (Keverne, 1983; Vandenburg, 1983).

Scalia and Winans (1975) investigated the projections of the MOB and the AOB in the rabbit, the opossum and the rat. Although slight species differences were found, their results confirmed the separate neural pathways of the AOB and the MOB in the three species investigated. Projections of the MOS arise from the olfactory receptors located in the olfactory mucosa of the nasal cavity and connect to the MOB. From the MOB, these projections run to the anterior olfactory nucleus, the olfactory tubercle, the pyriform cortex, the anterior cortical nucleus of the amygdala (C1), the posterolateral cortical nucleus of the amygdala (C2) and the entorhinal cortex. From the nuclei of the amygdala, the pathway of the MOS split, and efferents project to various termination points. From C1, efferents project to the pyriform cortex, the entorhinal cortex, subiculum, the amygdalo-hippocampal area, the posteromedial cortical nucleus of the amygdala, the medial nucleus of the amygdala, the bed nucleus of the stria terminalis (BNST) and the ventromedial nucleus of the hypothalamus (VMNH). Efferent projections from C2 to the anterior olfactory nucleus, the entorhinal cortex, the subiculum, the posteromedial cortical nucleus of the amygdala (C3), the medial nucleus of the amygdala (MNA), the endopiriform cortex,

agranular insular cortex and the olfactory tubercle (Keveter and Winans, 1981). The projections of the AOS originate in the sensory epithelium of the Vomeronasal organ, and projects to the AOB. From the AOB these efferents are projected through the lateral olfactory tract to the stria terminalis and the bed nucleus of the stria terminalis (BNST), the nucleus of the accessory olfactory tract (NAOT), the medial nucleus of the amygdala (MNA), and the posteromedial cortical nucleus of the amygdala (PMCNA) (C3). From the amygdala the pathway also splits, similar with the MOS. From the MNA the efferents project to the premammillary nucleus, the ventromedial nucleus of the hypothalamus, the medial preoptic area, the anterior hypothalamus, and back to the bed nucleus of the stria terminalis (BNST) and C3. From C3, efferents project to the bed nucleus of the stria terminalis, and back to the AOB. Thus, the two olfactory systems are anatomically completely separate, but run parallel with each other up to the lateral olfactory tract, after which they separate and terminate at separate cortical locations (Broadwell, 1975; Scalia and Winans, 1975; Davis et al, 1977; Keveter and Winans, 1981; Keverne, 1983; Meredith, 1983; Halpern and Martinez-Marcos, 2003)

The anatomical separation of the two olfactory systems indicates that the two systems have different functions. The main olfactory system is connected to portions of the brain that are responsible for the detection and recognition of volatile substances, while the accessory olfactory system is directly connected to the portion of the hypothalamus, which controls the release of reproductive hormones (Stoddard, 1976). This is an indication that in certain mammalian species the accessory olfactory system, with the Vomeronasal organ as its initial sensory apparatus, might play an important role in the modulation and control of reproductive behaviour. However, there is also a possibility that an interaction between the AOS and the MOS exists, since both systems are connected to MNA, C3 and VMNH (Winans et al, 1982). This interaction might explain phenomena like odoriferous memory and pregnancy block in mice. Winans et al (1982) cited the amygdala as the most probable location for interaction between the AOS and the MOS.

1.3.2 The physiological Relationship of the Vomeronasal System and the Hypothalamus

The hypothalamus is intricately involved with regulation of the reproductive processes by controlling the entire reproduction hormone cascade through the secretion of Gonadotropin Releasing Hormone (GnRH) (Swenson and Reece, 1993). Gonadotropin Releasing Hormone in turn stimulates the anterior hypophysis to release Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) into the blood stream (Bearden et al, 2004). These secretions exert their effect on the reproductive organs which in turn release their own secretions (mostly steroid hormones). These gonadal hormones have a negative feedback effect that provide hypothalamic control of GnRH secretion (Bearden et al, 2004) This feedback loop is one of many feedback pathways that exert some

control on GnRH secretion. The secretion of the luteinizing hormone releasing hormone (LHRH) from the parvicellular neurons of the hypothalamus entirely controls the secretion of LH from the gonadotrophs in the pars distalis of the hypophysis (pituitary gland) (Dellmann, 1976).

The hypothalamus lies ventral to the thalamus and constitutes the floor and the lateral wall of the third ventricle. It is considered to include the optic chiasma, the tuber cinereum, the mammillary bodies, the median eminence, the infundibulum and the neurohypophysis (Swenson and Reece, 1993; Bearden et al, 2004). The hypophysis consists of two main parts: the neurohypophysis (pars nervosa) which originates as an outgrowth of the brain and consist of nerve axon terminals from the magnocellular neurons of the supraoptic and paraventricular nuclei in the hypothalamus (Dellmann, 1976). The adenohypophysis originates as an outpocketing of the epithelial lining of the primitive mouth cavity and develops into the pars intermedia, pars distalis, and pars tubularis. The adenohypophysis is only functionally connected to the hypothalamus via the vascular hypophyseal portal system (Dellmann, 1976). The main reproductive secretions of the hypothalamus include Prolactin Releasing Factor (PRF), Gonadotropin Releasing Hormone (GnRH), Prolactin Inhibiting Factor (PIF) and Corticotropin Releasing Factor (CRH).

While the hypothalamus interacts with the neurohypophysis through its neural projections, it interacts with the adenohypophysis via the hypophyseal portal blood system. The hypophyseal portal vessels pass by the median eminence of the hypothalamus. At this point the hypothalamic hormones are secreted into the portal blood stream and carried to the adenohypophysis where they exert their effect on the secretions of the adenohypophysis. Gonadotropin Releasing Hormone (GnRH) reaches the Pars Distalis of the hypophysis through the hypophyseal portal blood system (Dellmann, 1976).

The biochemical pathways that cause the release of GnRH from the hypothalamic neurons gives further evidence for Vomeronasal organ involvement in reproductive modulation. The release of GnRH consists of a complex pathway where nitric oxide (NO) play a pivotal role (Dees et al, 2001);

1. NO release is stimulated by norepinephrine (NE) that binds to the α 1-adrenergic receptors on NO-neurons.
2. The stimulation of α 1-adrenergic receptors in turn increases the intracellular Ca^{2+} , which binds to calmodulin. The Ca^{2+} /Calmodulin complex activates an enzyme, NO-synthase, which increases NO secretion.
3. NO diffuses into the GnRH terminals and activates the enzymes guanylate cyclase (GC) and cyclooxygenase by increasing intracellular levels of Ca^{2+} .

4. The increase in Ca^{2+} activates the enzyme phospholipase 2 (PLA2).
5. Membrane phospholipids are converted to arachidonate by PLA2.
6. Arachidonate, in turn, is converted to prostaglandin E2 (PGE2) through cyclo-oxygenase.
7. PGE2 activates adenyl cyclase which increases cAMP.
8. cAMP activates protein kinase A which causes the exocytosis of GnRH granules into the hypothalamic portal blood system.

In addition, investigators have demonstrated that the cells responsible for the production and secretion of LHRH originate in the same portion of the embryonic nasal placode that also give rise to the Vomeronasal organ. These cells have been shown to migrate along a separate caudal branch of the vomeronasal nerve directly to the parvicellular portion of the hypothalamus (Quanbeck et al, 1997; Schwanzel-Fukuda and Pfaff, 1989; Wray et al, 1989; Yoshida et al, 1995; Yoshida et al, 1999). This area of the hypothalamus contains all the cells that give rise to Releasing Hormones that stimulate tropic hormone release from the Pars Distalis of the hypothalamus (pituitary gland) (Dellmann, 1976). Thus, a direct neural pathway from the Vomeronasal organ to the hypothalamic area responsible for producing releasing hormones has been established. Thus, there is a biochemical pathway for the secretion of GnRH from the GnRH neurons and a neural pathway that links the VNO with the hypothalamus. Clarke et al (2005) stained fibres of the BNST and the preoptic area (POA) with dopamine- β -hydroxylase (DBH) at three different stages during the oestrus cycle of ewes. Since DBH is secreted and transported with NE it follows that DBH staining would indicate NE secretion and activity. They found that neurons from the VMNH and the arcuate nucleus project to the BNST and the POA, even though little direct contact is made between neurons of the BNST and POA and GnRH neurons. It was confirmed that NE is released in the BNST and the POA, and especially in the lateral BNST (Clarke et al, 2005). In addition, NE activity fluctuates during the various stages of the oestrus cycle and increases during the follicular and preovulatory phase. Increased NE activity is thus associated with increased LH release, which coincides with increased GnRH release (Clarke et al, 2005). Since there is a neural connection from the VNO to the VMNH via the BNST. This indicates that an increase in NE activity could be precipitated by vomeronasal stimulation. This would activate the pathway described above and lead to increased gonadotropin secretion which would also explain the increase in reproductive performance after vomeronasal stimulation.

1.4 Pheromone Reception by the Vomeronasal Organ

1.4.1 Pheromones and the Vomeronasal System

Pheromones are non-volatile, high molecular weight molecules, usually carried in urine or other mammalian secretions. They elicit a definite behavioural response in individuals that comes into contact with these pheromones (Halpern and Martinez-Marcos, 2003). Rosa and Bryant (2002) showed that the pheromones present in the ram's wool and wax also stimulates the ewe's VNO. Originally the term "pheromone" was restricted to insects (Karlson and Butenandt, 1959), and defined as chemical signals secreted by one individual, which caused a definite, controlled response in a second individual. Two types of pheromones were recognized in insects: releasing pheromones and priming pheromones (Wilson and Bossert, 1963). Releasing pheromones are defined as pheromones that cause an immediate, but reversible, change in behaviour. Priming pheromones are defined as pheromones that initiate a chain of physiological events, either inhibitory or stimulatory, in which endocrine, reproductive and possibly other systems are altered (Izard, 1983). However, the direct application of this original definition in mammalian chemical communication is obscured by the variation in response to chemical cues of different individuals (Vandenburgh, 1983). Thus in mammals the term "signaling pheromone" is preferred instead of "releasing pheromone" (Bronson, 1982).

Several investigators have suggested that the VNO contains the receptors for large, non-volatile molecules (Wysocki and Wellington, 1980; Keverne et al, 1986), while the main olfactory neurons of the nasal cavity are sensitive to volatile molecules (Stoddard, 1976). Meredith (1983) cited that these physiological responses include acceleration of puberty in females, changes in oestrus-cyclicity, pregnancy block in mice and a testosterone response in male mice in the presence of female mice. The acceleration of puberty in mice has been extensively studied. The onset of puberty and vaginal opening in young female mice was hastened when these young females were exposed to male mouse urine (Meredith, 1983). First oestrus and vaginal opening occurred 20 days earlier in exposed females, compared to non-exposed females. The pheromone responsible for the acceleration of puberty in young female mice is dependent upon the androgen levels in the male (Castro, 1967; Vandenbergh, 1967). The urine from castrated males loses its potency 10 –15 days after castration, but regains potency about 60h after the administration of a single dose of 1.0 mg testosterone propionate (Castro, 1967; Vandenbergh, 1967). The urine from testosterone treated females has the same effect as urine from intact males and the expression of the androgen-dependant puberty accelerating pheromones is indirectly dependent upon the social environment (Vandenburgh, 1983). Two studies, quoted by Vandenburgh (1983) support this. In the first study, urine from dominant and subordinate male mice was used. Vandenburgh (1983) also found that only urine from dominant males accelerated oestrus in young females. Drickamer and Hoover

(1979) found that direct contact of pre-pubertal females with the urine of pregnant or lactating females, accelerated puberty. These findings are a strong suggestion that pheromones may form an important link between the behavioural and physiological mechanisms that control social dynamics. Pheromones are the messengers that convey information from one individual to another (Izard, 1983; Keverne, 1983; Dulac, 2000). For the transfer of information to be successful, the messenger molecule must first be detected, before its information can be extracted and utilized. As stated earlier, the AOS with the VNO as its primary sensory apparatus is the best candidate for detecting pheromones and relaying their information to the endocrine system. The possible involvement and role of the AOS in this mechanism was demonstrated in a study by Sanchez-Criado (1982). When female rats were raised in the absence of male rats or any male cues, the onset of puberty was delayed. As adults, these same females also expressed longer oestrus cycles. When exposed to males or male cues, puberty was accelerated and oestrus cycles were shortened. However, with peripheral deafferentation of the vomeronasal nerves the acceleration of puberty was prevented (Sanchez-Criado, 1982) as peripheral deafferentation renders the AOS non-functional. Pheromones can still be detected by the VNO, but information carried by the Vomeronasal organs' nerves cannot be relayed to the endocrine system.

The acceleration of puberty in the presence of males has been demonstrated in various domestic species such as pigs (*Sus scrofa*) (Brooks and Cole, 1970), cattle (*Bos taurus*) (Izard and Vandenberg, 1982) and in sheep (*Ovis aries*) (Ungerfeld, 2003). When female mice are housed in groups, puberty in young females is inhibited or delayed (Drickamer, 1974). Urine collected directly from the bladder of grouped and isolated females are equally potent in inhibiting puberty in young females. However, only the externally collected urine from grouped females inhibited puberty in young females (McIntosh and Drickamer, 1977). The mechanism involved is a complex system involving ovarian (Drickamer et al, 1978) and adrenal (Drickamer and Murphy, 1978) hormones that controls the secretion of a puberty inhibiting substance into the urine, and a urethral secretion that inhibits the activity of this puberty inhibiting substance. The social environment affects the urethral secretion, which is then present in the isolated female only and then mediated via pheromones (McIntosh and Drickamer, 1977). In the presence of female pheromones, the secretion of the urethral factor is inhibited ((McIntosh and Drickamer, 1977). Similar results have been reported in two other species of rodent, *Notomys alexis* (Breed, 1976) and *Peromyscus maniculatus* (Lawton and Whitsett, 1979) and in three species of primates, *Callithrix jacchus* (Abbott and Hearn, 1978), *Saguinus fuscicollis* (Katz and Epple, 1980) and *Meriones unguiculatus* (Payman and Swanson, 1980). The presence of adults of either sex also influences the onset of puberty in young male mice. Vandenberg (1971) reported that in general the presence of adult males inhibits the onset of puberty, while a female presence accelerates puberty.

Three behavioural phenomena have been observed in connection with the normal oestrus cycle of the female: enhancement, suppression and synchrony (McClintock, 1983). It has been reported that in certain species, oestrus is enhanced in all-female groups (McClintock, 1983). The oestrus cycle is shortened and the rate of ovulation is higher. The enhancement of the oestrus cycle in one individual is dependent on the phase of oestrus in which the majority of the group find themselves (McClintock, 1983). However, this effect is species dependant. In the guinea pig (*Cavia porcellus*) a shortening of the oestrus cycle is observed with exposure to periovulatory odours, while in the rat, the same result is achieved with odours from the follicular phase (McClintock, 1983). The suppression of oestrus has been reported in several species. Most work has been done on house mice (*Mus musculus*), but similar results have been reported in deer mice, *Peromyscus maniculatus* (Lombardo and Terman, 1980), Indian field mice, *Mus booduga* (Dominic and Pandey, 1979), and wild hopping mice, *Notomys alexis* (Breed, 1976; McClintock, 1983). Isolated adult female mice have oestrus cycles ranging from four to six days. The oestrus cycle in the isolated female is characterized by the absence of a functional corpus luteum, which cannot support implantation. A functional corpus luteum is only formed after the stimulation of mating. The oestrus cycle length is increased when adult females are grouped together, thus ovulation occurs less frequently (McClintock, 1983). This phenomenon has also been demonstrated in wild populations of mice. When females were removed from their family groups, their cycles shortened and they became more fertile (Crowcroft and Rowe, 1957). Pheromones deposited in urine are involved. Whitten (1985) demonstrated that when females were separated from each other and from their soiled bedding, the number of females with delayed cycles was halved. When the VNO was excised, the number of females with delayed cycles was also reduced.

Synchrony of oestrus cycles occurs when females are grouped together. This has been described for golden hamsters, *Mesocricetus auratus* (Handelmann et al, 1980), rats, *Rattus norvegicus* (McClintock, 1978), and humans (McClintock, 1971; Graham and McGrew, 1980; Quadagno et al, 1981). McClintock (1978) reported that individually housed female rats that shared the same recirculated air, had oestrus cycles that synchronized as quickly as those of females housed in groups. This suggests that smell provides enough stimulation to synchronize oestrus cycles in female rats. It was also demonstrated that odours emitted during the follicular phase, shortens and advances the cycles of other females, while odours emitted during the ovulatory phase, lengthens and delays the cycles of other females. Odours from the luteal phase have significant effect on the cycles of other females (Winfree, 1980). Very little work has been done to determine whether synchrony is mediated via the MOS or the AOS. Results supporting the volatile nature of the stimuli implicate the MOS, but the neural connections of the AOS to the hypothalamus indicate vomeronasal involvement (McClintock, 1983).

The introduction of males to a group of females is well known to induce synchrony. There is strong evidence that this is mediated through the AOS since exposure to male urine is a prerequisite for a response (McClintock, 1983).

It has been shown that female mice express a preference for bedding soiled by intact males, over bedding soiled by castrated males. It is the urine from intact males that carry the pheromones to which the females are sensitive (Keverne, 1983). Although female mice are not able to consciously discriminate between individual males, males different from the stud male do have an effect on the physiological state of the female (Keverne, 1983). If the stud male is from strain "A", and the female is exposed to the urine of male "B" early in pregnancy, she will abort spontaneously (Keverne, 1983). The same phenomenon is not observed if she is exposed to the urine of male "A" and this is known as pregnancy block (Bruce, 1960; Keverne, 1983). Selective lesioning of the MOB prevents the females to distinguish between the soiled bedding of intact and castrated males, but has no effect on the occurrence of pregnancy block (Bruce, 1960). The exact opposite is observed with selective lesions of the AOB. Pregnancy does not occur, but females can still distinguish between bedding soiled by intact and castrated males (Keverne, 1983).

Although most of these studies were done on mice and hamsters, a few studies have been done on sheep and goats, with dissimilar results. Cohen-Tannoudji et al (1989) exposed sexually experienced anoestrus ewes to male cues and reported a substantial increase in circulating LH in these ewes. They repeated the experiment three times, but destroyed vomeronasal input by Vomeronasal organ cauterisation in the second repetition, and vomeronasal nerve section in a third repetition. They found that neither of the procedures inhibited the rise in LH, thus they concluded that the VNO is not involved in the perception of ram odours or pheromones. In contrast, Booth and Webb (2011) demonstrated that by destroying the nasal incisive duct by cauterization, which was replaced by scar tissue, prevented substances from reaching the receptor cells in the VNO and resulted in a significant absence of the primary LH peak that is necessary for ovulation to occur. The physiological mechanisms and effects of the "male-effect" in sheep and goats has been well documented (Signoret and Lindsay, 1982; Over et al, 1990; Hamadeh et al, 2001; Abecia et al, 2002; Knights et al, 2002; Rosa and Bryant, 2002; Blaszczyk et al, 2004). Unfortunately, few of these studies relate the "male-effect" to vomeronasal function. On the other hand, Doving and Trotier (1998) indicated that after the VNO is stimulated, LHRH is released and in turn this promotes an increase in the level of LH, which in turn induces sexual behaviour.

1.4.1.1 Vomeronasal Receptor function

Molecular studies have confirmed that vomeronasal and olfactory receptors originate from different sets of genes. Olfactory receptors are encoded by a large set of about a thousand genes (Dulac, 2000) while three independent families of vomeronasal receptors (VRs) have been identified, V1R, V2R and V3R (Dulac, 2000; Pantages and Dulac, 2000; Zufall et al, 2008). These receptors are expressed in the VNO only, and are not related to olfactory receptors (ORs) (Dulac, 2000). Each family is expressed by specific non-overlapping sub-population of neurons and thus only one type of receptor is expressed by each neuron (Dulac, 2000; Liman, 2001; Zufall et al, 2008). Dulac (2000) suggests that the specific distribution of each type of VR plays a role in pheromone reception. Certain pheromones will stimulate only certain neurons (or certain sub-populations), and could help to distinguish between different pheromonal signals (Dulac, 2000). V1R and V3R neurons both express $G_{\alpha_{i2}}$ -protein and olfactory cell adhesion factor (OCAM) and are found lining the lumen, superficial to the V2R neurons, which expresses G_{α_0} -protein. Dulac (2000) also reports a number of studies that suggests that neural projections from these two sub-populations remains segregated up to the AOB. Projections from neurons expressing V1Rs and $G_{\alpha_{i2}}$ project mainly to the anterior AOB while V2R G_{α_0} neurons project to the posterior AOB. This suggestion is supported by Jia and Halpern (1996), who traced the axonal pathways of both sub-populations of receptor expressing neurons. The V1R family comprises about 80 genes (Zufall et al, 2008) and is distantly related to the mammalian bitter taste receptor T2R (Dulac, 2000). The V2R family, which consists of about 50-100 genes (Zufall et al, 2008), are characterized by a large, extracellular N-terminal binding domain, and are closely related to metabotropic glutamate receptors, Ca^{2+} sensing receptors and the putative taste receptors T1R1 and T1R2 (Dulac, 2000; Zufall et al, 2008). The V3R family consists of about 100-120 genes (Pantages and Dulac, 2000). Although the existence of three sub-populations of vomeronasal neurons, with different pheromone receptors, suggests that the AOS is divided into different units with related, but separate functions, there is little evidence to support this. Krieger et al (1999) suggests that $G_{\alpha_{i2}}$ -neurons are sensitive to lipophilic stimuli, while G_{α_0} -neurons are stimulated by “proteinaceous” stimuli.

1.4.2 The Vomeronasal Organ and Flehman

Flehman is a unique behavioural phenomenon in most ungulate species. Flehman is a prominent aspect of male sexual and social behaviour, and is usually performed in the presence of certain olfactory stimuli, such as freshly voided urine from a female individual or after investigation of the anogenital region of the female by the male. It is much less prominent in female social and sexual behaviour. During a typical flehman response, the head and neck is extended so that the head is in a horizontal position and the upper lip curled upward (Dagg and Taub, 1970).

The exact function of flehman is still uncertain, but most theories implicate either an olfactory or vomeronasal (thus chemosensory) connection to flehman behaviour (Knappe, 1964; Bailey, 1977; Ladewig and Hart, 1980). In these theories flehman is considered to play an important role in especially the transport of odourants into the vomeronasal lumen. According to Doving and Trotier (1998) it is believed that fluid-borne chemical stimuli, such as sex pheromones, are transported by flehman from the oral cavity to the Vomeronasal organ. Knappe (1964) postulated that flehman and the VNO are intimately associated with each other, and that flehman aids the transport of vomeronasal stimuli to the VNO. He suggested that the nasal opening of the nasopalatine duct enlarges during the curling of the upper lip, while the horizontal position of the head aided the flow of secretions, which contain odourants, into the VNO.

Ruminants have an incisive papilla and the openings of the naso-incisive ducts are located on the hard palate just behind the dental pad (Doving and Trotier, 1998). This flow of secretions would be aided by the establishment of a negative pressure within the VNO lumen, due to the shrinkage of erectile tissue surrounding the lumen. Dagg and Taub (1970) rejected Knappe's (1964) theory and suggested that the lip-curl closed the nostrils and trapped odour-filled air in the nasal cavity. This enables the animal to investigate trapped odours for extended periods of time by normal olfaction. Bailey (1977) also suggested a possible mechanism with regard to the ring-tailed lemur (*Lemur catta*). In the ring-tailed lemur stimulus substances are deposited in the mouth, close to the buccal openings of the nasopalatine ducts. Vasoconstriction of the blood vessels within the VNO causes an increase in lumen volume and thus a negative pressure within the VNO lumen and this sucking action draws stimulus substances into the VNO. In a small animal such as the ring-tailed lemur, these two mechanisms could prove to be sufficient to transport stimulus substances into the VNO.

In larger animals these two mechanisms could prove to be insufficient, however Estes (1972) suggested one possible mechanism of the flehman response that would ensure significant transport of stimulus substances into the VNO. If the nares are closed during inspiration, and the base of the tongue and the velum closes the rear of the oral cavity at the same time, the contents of the nasopalatine ducts will be drawn upwards towards the nasal cavity. Together with this, fluids containing stimulus substances will be pulled into the nasopalatine duct and into the VNO. Alternatively the nares might be narrowed to slits. During inspiration a rapid stream of air would move across the nasal opening of the nasopalatine duct and this would draw the contents of the nasopalatine ducts upward by way of the venturi effect (Bailey, 1977). Bailey went further and gave a partial explanation of flehman in the ring-tailed lemur. Volatile stimuli are detected through normal olfaction. This stimulates the behaviour of "lapping" (a rapid licking action in which the head is kept still and only the tip of the tongue is in contact with the stimuli), which transfers non-volatile stimuli to the buccal openings of the

nasopalatine ducts. Through diffusion into the nasopalatine duct, a small fraction of the non-volatile stimuli reach the VNO and initiates the flehman response. By vasodilation of the vomeronasal blood vessels, fluid in the VNO is pumped out into the adjacent part of the nasopalatine duct while the nares are narrowed and accompanied by rapid inspiration. This causes the flow of a strong air current across the nasal opening of the nasopalatine ducts. The fluids within the nasopalatine ducts, together with those expelled from the VNO, are drawn upwards and replaced by fluids from the lower part of the nasopalatine duct, which contains the non-volatile stimulus substance. Vasoconstriction of the vomeronasal blood vessels causes a vacuum in the VNO lumen and pulls the fluids containing the stimulus into the VNO. He limits this mechanism to species that can perform the “lapping” behaviour. Ladewig and Hart (1980) used sodium fluorescein dye to show that fluid material can pass through the nasopalatine duct, with or without flehman. Dye was found in the posterior part of the VNO only in those individuals in which flehman were observed. This is the part where the chemosensory epithelium is situated. They postulated that flehman must therefore, through some mechanism, aid in the transport of fluids from the anterior to the posterior end of the VNO.

The investigations of Ladewig and Hart (1980) brought them to a series of conclusions concerning the relationship between flehman and VNO function:

1. Flehman is stimulated by an olfactory stimulus.
2. Flehman facilitates the passage of fluids from the oral cavity through the nasopalatine duct and into the posterior VNO.
3. Flehman is not necessary for oestrus detection but is important in the detection of di-oestrus and possibly early pro-oestrus. Oestrus can be detected by normal olfactory function.
4. VNO stimulation helps to maintain a male’s sexual interest and enhances the probability of further copulation after the initial post ejaculatory interval.
5. The close investigation of urine and the uptake of urine samples into the oral cavity facilitate vomeronasal investigation and are not associated with gustatory function.

1.5. Outline and aim of this study

In sheep it is well known that the ram plays a big role in triggering reproductive cycling in anoestrus ewes (Booth and Katz, 2000; Abecia et al, 2002; Clarke et al, 2005; Abass et al, 2012). The overall aim of this study was to gain further knowledge of the reproductive response of does after the introduction of bucks and the system(s) directly responsible for this response. This includes the investigation of the specific role of vomeronasal stimulation in the “buck” effect. Vomeronasal stimulation was controlled by occluding the vomeronasal ducts, which prevented chemical stimuli from interacting with the vomeronasal sensory epithelia, thus preventing primary stimulation to occur. This differs from previous studies where chemical and physical methods were used to cut the neural pathways linking the accessory olfactory bulb with the hypothalamus thus blocking the neural transduction of the primary stimulus to the hypothalamus. The methods employed in this study was followed because evidence suggests that there is an alternative and direct neural pathway from the VNO to the specific portion of the hypothalamus responsible for the production and release of various reproductive releasing factor hormones (Raisman, 1972; Cohen-Tannoudji et al, 1989; Davis et al, 1997; Booth and Katz, 2000; Halpern and Martinez-Marcos, 2003). This implies that in previous studies where the main pathway was severed either by physical or chemical means, that all contact between the AOB and the hypothalamus was not completely interrupted and therefore a hypothalamic response was still possible with vomeronasal stimulation.

With the ongoing pressure that small stock farmers undergo from external factors, it might be necessary to exploit as much natural management practices as possible to be more sustainable and profitable. It is also true that many organisations and groups are against artificially produced hormone administration to synchronize females for reproduction. By discovering and applying the natural factors responsible for the “male-effect”, it will be possible to overcome the administration of artificially produced hormones to synchronize female animals for reproduction and together with improved husbandry, it may be possible to increase the reproductive rate from three cycles in two years to two reproductive cycles per year.

Since the VNO might play a critically important role in mammals during reproduction and offspring recognition, this study was initiated to investigate the influence the VNO may have on reproduction success on a small stock farm. If the VNO plays a role on reproduction in small stock, many farming enterprises could benefit from knowing the workings of the VNO and applying the management practices associated with these workings of the VNO.

Chapter 2: Materials and Methods

This research was approved by the Animal Use and Care Committee (AUCC) to comply with essential research procedures. The trial was conducted during the late spring and early summer in the southern hemisphere (November). The trial was carried out at the University of Pretoria's experimental farm which is situated in Hatfield, Pretoria, South Africa. The average annual rainfall for Pretoria is 517mm, it lies 1305m above sea level and the mean temperature is around 17°C with high's above 35°C and lows can be near freezing in some years. Temperatures during the experimental period varied between 6°C and 22°C, with a total of 5mm of rain for the trial period.

2.1 Animals

Twenty-one indigenous, multiparous does, three years of age, were used in this study. The does were raised at the Mara Research Station in the Limpopo Province, where they were kept at range on natural sweetveld pastures. Data from the station indicated that all does had at least two successful breeding seasons. The goats were transported from the Mara Research Station to the Experimental farm at the University of Pretoria (Pretoria, Gauteng Province) one month before the onset of the trial to ensure sufficient adaptation. On arrival the goats were kept in a boma (an enclosure designed for game), completely separate from all other animals. This meant that the animals could not interact in any way with any other animals. Four does had newly born kids when they arrived on the farm.

The animals were randomly divided into two groups according to bodyweight: a treatment group (Group 1, 11 animals), and a control group (Group 2, 10 animals). The measured bodyweights were arranged in a descending order. Every second animal was assigned to the treatment group, with the remainder assigned to the control group. The two groups were kept together to minimize any difference in treatment. The animals were identifiable by numbered ear tags.

2.2 Treatment

The animals in the treatment group (Group 1) had their Vomeronasal organs rendered non-functional by surgical cauterization. The surgical procedure was conducted in the small stock surgical rooms at the Onderstepoort Academic Veterinary Hospital in the Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Republic of South Africa. All does were anesthetized by intravenous administration of thiopentone at a dose of

15mg per kg body weight. The does in the control group had their nasal cavities flushed with physiological saline while under anaesthesia.

Cauterization of the VNO:

The cauterizing forceps were introduced into the nasopalatine ducts from the oral openings and positioned as close as possible to the nasal openings of the ducts. The oral openings of the nasopalatine duct are on either side of the incisive papilla, just caudal to the dental pad. The nasopalatine ducts were cauterized by slowly withdrawing the forceps during the cauterization process. During the recovery process, the entire duct is replaced with “scar-tissue” and the nasal and oral openings of the duct are covered over by mucous epithelial tissue of the nasal and oral cavities, respectively. Thirty days after this procedure, occlusion of the ducts were evaluated by physical observation of the duct openings. This procedure completely occluded both the oral and the nasal openings of the incisive ducts and rendered the VNO non-functional (Booth and Katz, 2000).

Oestrus synchronization of does:

The does were weighed and scanned for pregnancy before synchronization. The does were synchronized by two injections of Estrumate® (0.5 ml per doe), ten days apart (the first injection on day 1, and the second on day 10 of the trial) and by inserting Ovakron sponges vaginally, containing 40 mg flugestone acetate obtained from DNAfrica Anipharm (Pty) Ltd. Normal application of sponges includes insertion of sponges or tampons on day 1, removal on day 14 with PMSG treatment. For this trial sponges were inserted on day 1 and removed on day 12. PMSG was not used as PMSG stimulates follicle development to produce multiple lambs, and this was not part of this study.

Flugestone acetate is a synthetic progestagen with progesterone like effects. It inhibits oestrus and ovulation in treated does, while removal has a “rebound” effect resulting in FSH release and normal follicle development, oestrus and ovulation (Kasikci et al, 2011). Estrumate® (cloprostenol) is a luteolytic agent and causes the regression of the corpus luteum. However it is only effective on the corpus luteum that is at least 5 days old, due to the lack of sufficient prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors in the premature corpus luteum (Patterson et al, 2004). Ideally the corpus luteum should be older than 7 days. The typical length of the oestrus cycle in does is 21 days (Bearden et al, 2004). Oestrus should be detected 36 to 72 hours after the administration of Estrumate®. Due to the insensitivity of the premature corpus luteum during the first 5 days of the oestrus cycle only about 60% to 65% of treated animals will respond to a single treatment (only 60% to 65% of animals will have corpora lutea older than 5 days). By repeating the treatment 10 days later those animals that did not respond would have corpora lutea that is at most 10-15 days old, and those animals that did respond to the first

treatment would have corpora lutea that is at least 7-9 days old. All animals would then be expected to respond to the second treatment, since all corpora lutea would be older than 5 days. All animals should then exhibit oestrus 36-72 hours later, thus on day 11 to 13. The animals were allowed to rest for twenty-one days, the length of a typical oestrus cycle. Oestrus was expected on day 32 to 34.

Table 2.1 Oestrus synchronization of does in VNO trail

	Day	
Intravaginal sponges	0	Estrumate Injection
	4	
	8	
	10	Estrumate Injection
	12	21 day oestrus cycle
16		
20		
24		
28		
Heat	32	Bucks introduced
	33	
	34	
	35	
	36	Bucks taken out
Pregnancy scan	72	

Introduction of bucks and breeding:

The bucks were introduced on the day of expected oestrus, day 32, and remained with the does up to the end of the trial (day 36). As mentioned earlier, all the does (treatment and control) were kept together to ensure equal exposure to the bucks.

The animals were kept outdoors during the first 26 days of the trial, while being handled regularly to accustom them to the frequent handling to which they would be exposed to during the trial. On day 27 (the start of blood collection) the animals were moved indoors to make handling easier and to minimize stress. For the remainder of the trial they were kept in a large barn with large windows incorporated into the roof. This ensured that the animals were exposed to enough natural light to minimize the effect of changing daylight on the reproductive performance of the does after being moved indoors. No artificial lighting was used at any stage during the trial, except when blood sampling was done at night. To minimize stress throughout the trial, handling between blood collection sessions was restricted to a minimum. The does were scanned for pregnancy 40 days after the bucks were taken out.

2.3 Feed

After arrival the does received milled hay, (*Digitaria eriantha* subsp. *eriantha*), which is produced on the experimental farm, with a small amount of concentrate. They also had free access to fresh water. After vaccination on the next morning, the animals were adapted to a commercial, pelleted total mixed ration (Senwesko Animal Feeds) over a period of 14 days. The animals were not allowed to graze during this 14 day adaptation time. The animals were fed according to their body weight. During the trial period feed intake was calculated as 2% of average bodyweight to compensate for lactating does. Predicted feed intake was calculated as 532g per day and then feed was rounded upward to 550g per doe per day. Milled hay and water was freely available throughout the trial. The bucks followed an identical feeding regime before being introduced to the does on day 32.

2.4 Medication

The animals were processed one day after arrival. They were treated with a broad spectrum anti-parasitic treatment (Ivomec[®], Merial Animal Health) and a pour-on dip against tick infestations (Deadline[®], Bayer Animal Health) according to the respective instructions of each product. They were vaccinated against pulpy kidney and pasteurellosis. The animals were also weighed and scanned for pregnancy. The initial weights were used to

stratify animals according to weight and to randomly assign does to either the treatment or the control group. The does were weighed again before the onset of the trial (thus they were weighed twice). One animal contracted pasteurellosis shortly after arrival and died. A second animal suffered a broken horn after a severe bunt and was treated with injectable penicillin, Duplocillin (Intervet) and a topical spray, Wound Sept (Obaron Laboratories Pty Ltd.). Due to the short time span between arrival and the onset of the trial, the does could not be vaccinated against Blue Tongue, although this would have been a good practice.

2.5 Blood collection

Blood sample collection began five days before the bucks were introduced, thus on day 27, and continued for ten days, up to day 36. Blood samples were taken twice daily (08:00 and 20:00) for the first four days (up to day 30). On day 31 blood samples were taken every 4 hours for a period of 12 hours. During the following 24 hours blood samples were taken every 2 hours, and during the next 12 hours blood samples were again taken every 4 hours. Thus the frequency of blood sampling increased during the 48 hours (days 31 and 32) in which oestrus was expected (as is illustrated in Table 2.2). During the next 4 days (days 33-36) blood samples were again taken twice daily at 08:00 and 20:00 (see Table 2.2). Blood samples were taken via jugular venipuncture, by using 18G needles. Blood were collected in, and stored in Lithium-Heparin tubes (Vacutainer 10ml). After collection the blood samples were immediately put on ice. Within at least one hour after collection the blood samples were centrifuged for 7 minutes. Some samples needed to be centrifuged for a further 3 minutes before a clear supernatant could be extracted. In a small percentage of samples haemolysis occurred. The supernatant fluid (blood plasma), was extracted by 5 ml disposable pipettes (Merck). A new pipette was used for every sample to avoid contamination. Plasma was transferred, stored, and frozen at -15°C in 2ml Eppendorff tubes (Merck). After the blood sample collection on day 32, the bucks were introduced and all does serviced by bucks were recorded. Recordings were done by accurate recording of animal identification (ear tags).

Table 2.2 Demonstrating day-by-day activities of the trial

Day	Activity	Time 24h
1	Trial commences; Estrumate injection 1	12:00
	Intravaginal sponges inserted	12:00
2-9	Rest	
10	Estrumate injection 2	12:00
12	Intravaginal sponges taken out	12:00
12-25	Rest	
26	Animals are moved indoors	
27-30	Blood collection commences	08:00
		20:00
31	Blood collection	08:00
		12:00
		16:00
		20:00
		22:00
32	Blood collection	00:00
		02:00
		04:00
		06:00
		08:00
		10:00
		12:00
		14:00
32	Introduction of buck and continuing of blood sampling	16:00
		18:00
		20:00
		22:00
33	Blood collection	00:00
		04:00
		08:00
		20:00
34-36	Blood collection	08:00

2.6. Statistical analysis

The effect the Vomeronasal organ has on LH, Estradiol and Cortisol secretions was analysed using multifactorial analysis of variance (MANOVA) by means of the General Linear Model (GLM) procedure of SAS (2003). Time was the only variable. Hormone levels were measured by means of Beckman Coulter Immunoassay System (a paramagnetic particle, chemiluminescent immunoassay).

Data was recorded in Microsoft Excel (2013). The results were statistically analysed by analysis of covariance. Significance was tested at a level of 95% certainty ($P \leq 0.05$).

Chapter 3: Results and Discussion

3.1 Results

The results after cauterization of the Vomeronasal organ in does on the plasma concentrations of estradiol, LH and cortisol are presented in Table 3.1. Estradiol is measured in pmol/L, LH is measured in IU/L and cortisol in nmol/L. When considering the values within these tables it is important to notice that the males were introduced at T=3. In Table 3.1 it is illustrated that T₁ is 8 hours before the rams are introduced at T₃; T₂ is 4 hours before the rams are introduced; T₃ is at ram introduction; T₄ is 4 hours after ram introduction; T₅ is 6 hours after ram introduction; T₆ is 8 hours after ram introduction; T₇ is 12 hours after ram introduction; T₈ is 16 hours after ram introduction; T₉ is 28 hours after ram introduction.

Table 3.1 Average values (\pm SD) for estradiol, LH and cortisol of both groups (Control (C) and Treatment (T)) during the different period intervals (T₁– T₉).

		Ave	\pm Std Dev	Ave	\pm Std Dev	Ave	\pm Std Dev
C	T1(-8h)	117,63	\pm 15,24	0,18	\pm 0,00	19,66	\pm 15,38
C	T2(-4h)	190,44	\pm 75,21	0,20	\pm 0,00	21,99	\pm 14,44
C	T3(0h)*	187,31	\pm 74,73	0,20	\pm 0,00	30,94	\pm 38,81
C	T4(+4h)	80,96	\pm 14,28	0,35	\pm 0,15	29,20	\pm 24,43
C	T5(+6h)	100,17	\pm 21,42	0,39	\pm 0,21	32,65	\pm 26,75
C	T6(+8h)	80,57	\pm 9,52	0,32	\pm 0,14	20,45	\pm 17,46
C	T7(+12h)	98,09	\pm 17,14	0,20	\pm 0,01	42,64	\pm 36,01
C	T8(+16h)	94,29	\pm 15,23	0,40	\pm 0,26	41,76	\pm 42,04
C	T9(+28h)	57,16	\pm 14,26	0,50	\pm 0,13	22,47	\pm 21,09
Average		111,85		0,31		29,12	
T	T1(-8h)	120,47	\pm 15,23	0,18	\pm 0,00	27,78	\pm 20,40
T	T2(-4h)	167,59	\pm 33,32	0,20	\pm 0,00	44,34	\pm 15,46
T	T3(0h)*	168,54	\pm 33,32	0,20	\pm 0,00	42,46	\pm 18,93
T	T4(+4h)	92,38	\pm 14,28	0,20	\pm 0,00	34,82	\pm 27,42
T	T5(+6h)	80,48	\pm 11,91	0,19	\pm 0,00	52,24	\pm 25,42
T	T6(+8h)	104,76	\pm 10,47	0,17	\pm 0,00	51,40	\pm 30,59
T	T7(+12h)	94,76	\pm 17,61	0,20	\pm 0,03	70,37	\pm 32,04
T	T8(+16h)	78,58	\pm 6,66	0,22	\pm 0,05	41,17	\pm 20,62
T	T9(+28h)	44,81	\pm 7,01	0,55	\pm 0,17	37,01	\pm 21,16
Average		105,82		0,23		44,62	

*T₃ (0h) – introduction of bucks

Table 3.2 Significance of the difference (P) between the average values of the treatment (T) and control (C) groups are given for estradiol, LH and cortisol.

Time	ESTRADIOL			LH			CORTISOL		
	C	T	P	C	T	P	C	T	P
T1(-8h)	117,63	120,47	0,34	0,18	0,18	0,11	19,66	27,78	0,16
T2(-4h)	190,44	167,59	0,20	0,20	0,20	0,19	21,99	44,34	*0,01
T3(0h)**	187,31	168,54	0,24	0,20	0,20	0,15	30,94	42,46	0,23
T4(+4h)	80,96	92,38	*0,05	0,35	0,20	*0,01	29,20	34,82	0,32
T5(+6h)	100,17	80,48	*0,01	0,39	0,19	*0,01	32,95	52,24	0,06
T6(+8h)	80,57	104,76	*0,00	0,32	0,17	*0,01	20,45	51,40	*0,01
T7(+12h)	98,09	94,76	0,34	0,20	0,20	0,50	42,64	70,37	*0,04
T8(+16h)	94,29	78,58	*0,00	0,40	0,22	*0,02	41,76	41,17	0,48
T9(+28h)	57,16	44,81	*0,01	0,50	0,55	0,23	22,47	37,01	0,07

* Means differed $P \leq 0.05$ and shows significance.

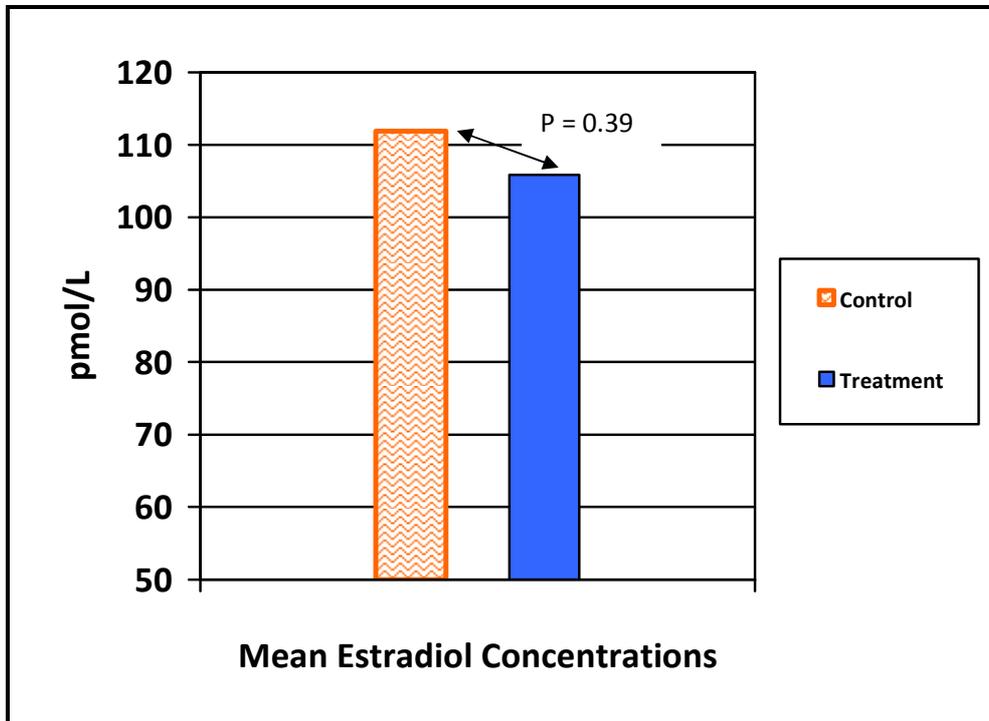
** T₃ (0h) – Buck introduction

Effect of VNO cauterization on Estradiol:

Graph 3.1 demonstrates that the overall average of estradiol concentrations in the control group were numerically higher than estradiol concentrations in the treatment group (111.85 IU/L vs 105.82 IU/L), however this difference was not significant ($P = 0.39$). This is also reflected in Graph 3.2 where a gradual increase in the difference between Est_C and Est_T can be observed.

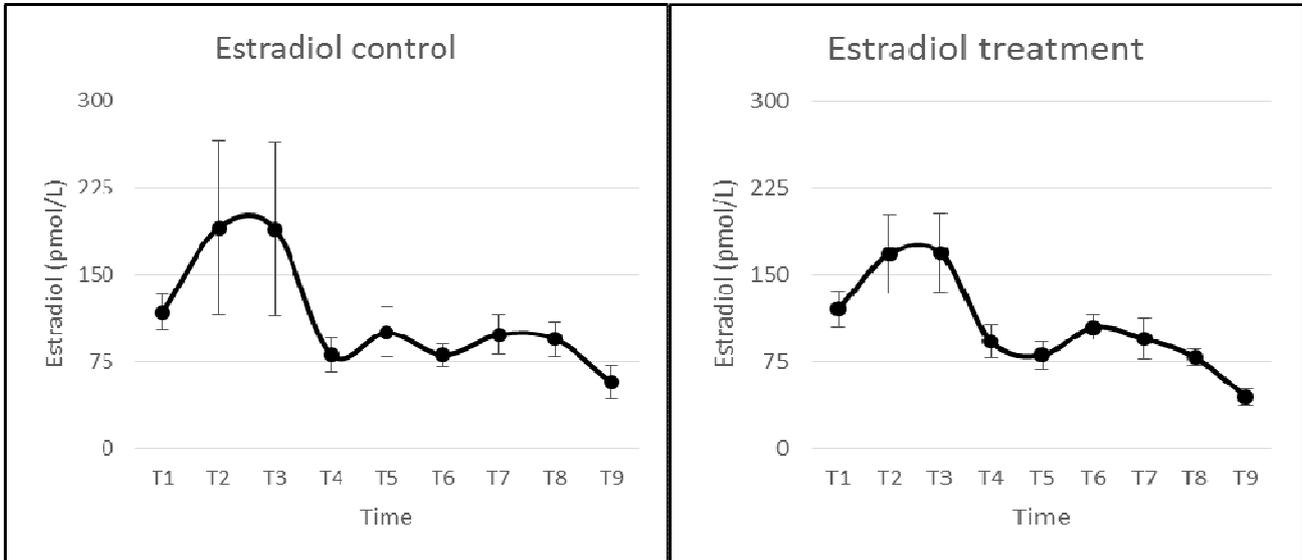
Estradiol concentration in the control group (Graph 3.2) started off at a point close to the overall average and reached a peak at T₂ (190.44 pmol/L) and held this high value at T₃ (187.31 pmol/L). After this the values declined sharply to T₄ (80.96 pmol/L) and showed a small increase in value at T₅ (100.17 pmol/L). From T₆ to T₉ the values for estradiol concentrations stayed low with the lowest value at T₉ (57.16). Estradiol concentration values in the treatment group followed a similar pattern to that of the control group, with peaks at T₂ (167.59 pmol/L) and at T₃ (168.54 pmol/L) after which it declined sharply to T₄ (92.38 pmol/L) and T₅ (80.84 pmol/L). As with the control group, estradiol concentrations for the treatment group also hits a low at T₉ (44.81 pmol/L).

Graph 3.1 Average estradiol concentrations in control (Est_C) and treatment (Est_T) groups for the trial period.



In general the graphs of Est_C and Est_T share a common shape, peak at the same intervals and hit the lowest value at the same interval, T_9 . The only main difference is that the control group showed a small increase at T_5 and again at T_7 , where the treatment group only increased a little at T_6 and decreased after that point. Table 3.2 shows that there were significant differences ($P \leq 0.05$) in estradiol values for the control and treatment groups. Beginning at T_4 , just after male introduction, the differences became significant ($P = 0.05$) and the differences remained significant at T_5 ($P = 0.01$), T_6 ($P = 0.00$), T_8 ($P = 0.00$) and T_9 ($P = 0.01$).

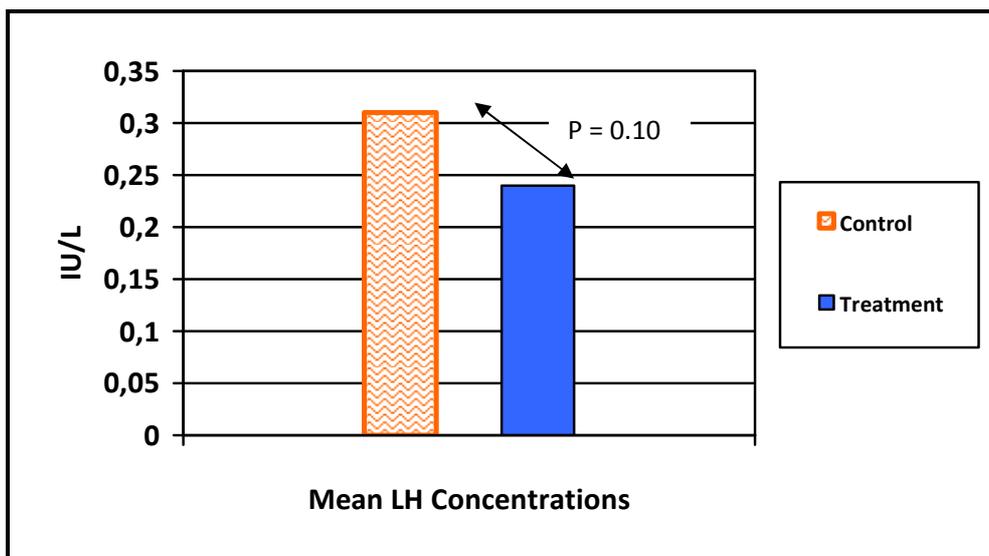
Graph 3.2 Average estradiol concentration in control and treatment groups displayed over time, with standard deviations also indicated for each group (T₁– T₉).



Effect of VNO cauterization on LH:

Graph 3.3 demonstrate that over the entire trial period luteinizing hormone levels in the control group (0.31 IU/L) were numerically higher than luteinizing hormone levels in the treatment group (0.23 IU/L). This difference in averages was however not significant (P = 0.10).

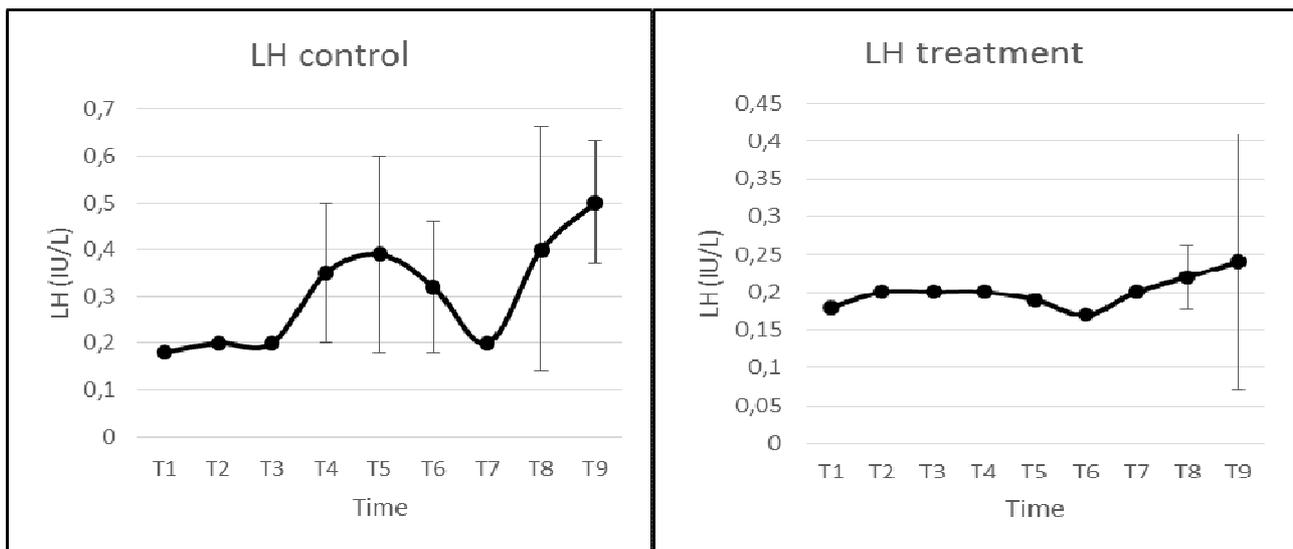
Graph 3.3 Average LH concentration in control (LH_c) and treatment (LH_t) groups for the entire trial period.



Graph 3.4 indicates that luteinizing hormone levels were constantly higher in the control group than luteinizing hormone levels in the treatment group. Graph 3.4 also shows that the LH_c group had two responses. The LH values increased from T₃ (0.20 IU/L) to T₄ (0.35 IU/L), stayed high at T₅ (0.39 IU/L) and at T₆ (0.32 IU/L). LH_c dropped to 0.20 IU/L at T₇, increased again at T₈ (0.40 IU/L) and peaked at T₉ (0.50 IU/L). The LH values for the treatment group varied very little over the trial period. From Graph 3.4 it can be seen that the treatment group demonstrated a small increase of LH from T₆ (0.17 IU/L) to T₇ (0.20 IU/L) and T₈ (0.22 IU/L) which peaked peaks at T₉ (0.24 IU/L).

As with estradiol, the average LH values at each interval between the two groups (Table 3.2) became significant just after male introduction at T₃ and the difference in LH concentrations remained significant at T₄ (P = 0.01), T₅ (P = 0.01), T₆ (P = 0.01) and T₈ (P = 0.02).

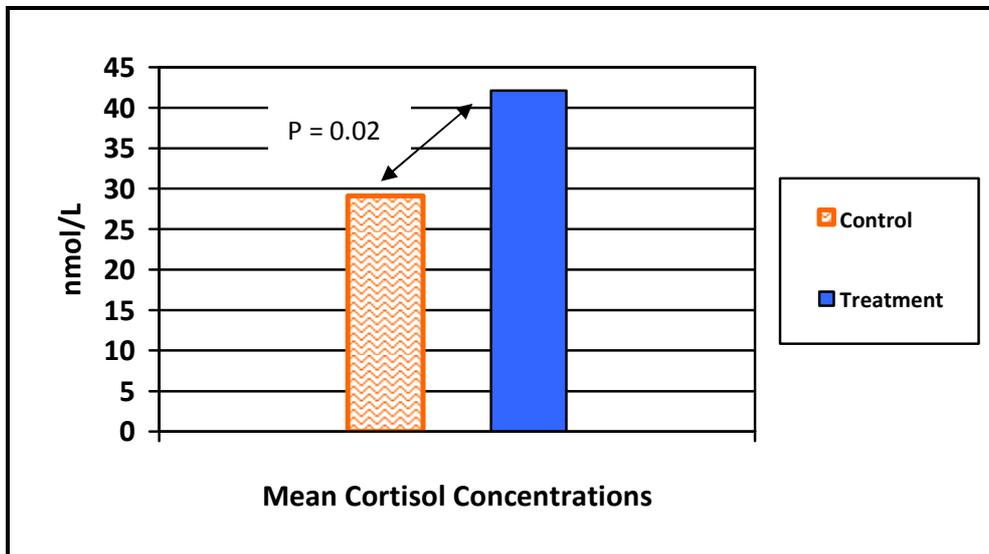
Graph 3.4 Average plasma LH concentration in control and treatment groups displayed over time, with standard deviations also indicated for each group (T₁– T₉).



Effect of VNO cauterization on Cortisol:

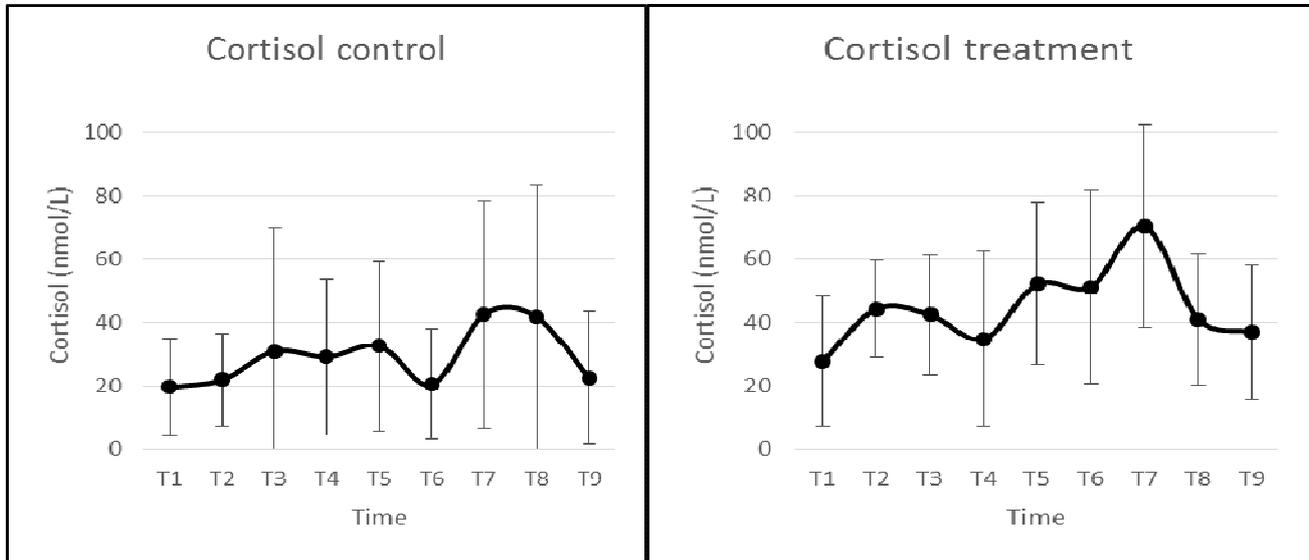
As is demonstrated in Graph 3.5, cortisol levels in the treatment group had a higher average (44.62 nmol/L) than cortisol levels in the treatment group (29.12 nmol/L), which is also reflected in Graph 3.6. This difference in the average cortisol values over the trial period was significant, with P = 0.02.

Graph 3.5 Average cortisol in control (Cort_c) and treatment (Cort_t) groups for the trial period.



Graph 3.6 shows that cortisol levels in the control group increased from T₁ (19.66 nmol/L), peaked at T₃ (30.94 nmol/L) and T₅ (32.65 nmol/L) with a slight depression at T₄ (29.20 nmol/L) just after the introduction of the males at T₃. At T₆ (20.45 nmol/L) Cort_c is almost at the base level and peaks again at T₇ (42.64 nmol/L) before declining sharply from T₈ (41.76 nmol/L) to T₉ (22.47 nmol/L). The cortisol levels in the treatment group had a more gradual increase than Cort_c from T₁ (27.78 nmol/L) to T₇ (70.37 nmol/L) with fair depressions at T₄ (34.82 nmol/L) and T₆ (51.40 nmol/L). From T₇ (70.37 nmol/L) Cort_t declines to T₈ (41.17 nmol/L) and T₉ (37.01 nmol/L). Although cortisol values for the treatment group are higher than the control group at every time interval except at T₈, the average cortisol concentrations only differed significantly at T₂ (P = 0.01), T₆ (P = 0.01) and T₇ (P = 0.04) (Table 3.2).

Graph 3.6 Average Cort_c and Cort_t, displayed over time, with standard deviations also indicated for each group (T₁ – T₉).



3.2 Discussion

Estradiol:

High concentrations of estrogens plays a critical role in behavioural signs of oestrus in females. As oestrus approaches, the maturing follicles secrete high levels of estrogens, which are needed for exhibition of sexual receptivity and initiation of hormonal changes that lead to ovulation (Bearden et al, 2004). Low concentrations of estrogens are inhibitory and very important in regulating the tonic release of gonadotropins, where high concentrations stimulate the release of GnRH, LH, FSH and other hormones (Bearden et al, 2004). High estradiol levels stimulates GnRH secretion and this in turn increases LH concentrations. LH is necessary for the formation of a functional corpus luteum which produces progesterone and brings about a decrease in estradiol concentration (Bearden et al, 2004).

Graph 3.2 indicates that the shape of estradiol levels in the two groups follow a similar pattern, but with numerically different amplitudes. Estradiol concentrations in the control group show a small increase at T₅ and this correlates with the small decrease in luteinizing hormone concentrations in the control group from T₅ to T₆ (Graph 3.4). Estradiol levels in the treatment group showed a small increase at T₆ and then a steady decrease from T₇ to T₉, which again correlates with a low LH_t level at T₆ and a small increase in LH concentrations from T₇-T₉ (Graph 3.4). Ungerfeld et al (2003) illustrated that estradiol levels in ewes that came into heat were higher to

those ewes that didn't show heat, as was also demonstrated in this study. This data demonstrated that the VNO has a definite influence on estradiol levels just prior to oestrus. The normal estradiol concentrations in both groups show that the physiological response of the ovaries in both groups were normal and that there were no differences in one group over the other. The sudden decrease in estradiol concentrations for both groups after T_3 clearly indicated that certain physiological disturbances took place after the introduction of the males. Graphs 3.2 and 3.4 also demonstrate that at T_3 (bucks are introduced at this point) estradiol concentrations peaks and LH concentrations were at a low. At T_4 , in the control group, estradiol concentrations decreased drastically (Graph 3.2) and LH concentrations increased to peak at T_5 (Graph 3.4). These correlations are normal for estradiol and LH concentrations just prior and just after ovulation (Bearden et al, 2004).

Luteinizing hormone:

The normal effect of male presence on cycling does is synchronization of oestrus, while anoestrus does return to oestrus. This increase in reproductive function is an effect of the hormonal changes instituted by the male on the female, thus the introduction of a male to a group of females will be reflected endocrinologically by the females. This effect is not only reflected in fluctuations and secretion patterns of the respective reproductive hormones, but also how they change in relation to each other (Bearden et al, 2004).

The difference in LH concentrations between the control group and the treatment group is shown in Graphs 3.3 and 3.4. Luteinizing hormone concentrations in the control group showed an increase in concentration at T_4 - T_6 with another increase at T_8 . LH normally have two peaks before oestrus, one in pro-oestrus and then again near the start of oestrus (Bearden et al, 2004). Thus, two LH peaks are necessary for females to ovulate and to reproduce successfully, as was demonstrated in the control group. The values for LH_c therefore exhibited a normal curve for LH and this indicate that the does responded normally to the introduction of bucks. There was a statistically significant ($P = 0.01$) absence of any increase in LH concentrations at T_4 - T_6 with only one visibly small increase in concentration at T_7 - T_9 . This indicates that the inactivation of the VNO had a consistent effect on the production and release of LH in the treatment group when compared to the control group. Failure to show heat and to become pregnant, as demonstrated in the treatment, group also show that this increase in LH concentration is important at pro-oestrus and also near the start of oestrus itself. The results of the treatment group indicate that there was no response in LH concentration after the introduction of bucks. The difference observed in LH secretion patterns indicates different physiological responses to the male presence between the groups. This is also supported by the changing trend observed in Graph 3.4. A definite increase in LH concentration in the control group is visible after T_3 which was not seen in the treatment group. From the

graphs for LH (Graphs 3.3 and 3.4) there is an indication that impaired VNO function does have some effect, at least on the pattern of LH secretion necessary for ovulation.

Graphs 3.2 and 3.4 also show that there is a difference in relationship of estradiol and LH values in the control group where a typical feedback mechanism can be identified with Est_c values decreasing one period before LH_c shows an increase. There is also a difference in relationship between LH_T and Est_T , where a delayed feedback is noticeable. LH_T only shows a small increase after the second time that Est_T decrease in value, at T_7 . Feedback is delayed to such a point, that the feedback mechanism fails to keep the secretion of the estradiol and LH sufficiently synchronized so as to ensure reproductive success. The pattern of Est_T and LH_T is obscured to such an extent that there is no relation between the two graphs and they fluctuate independent of each other.

These observations show that LH is the primary hormone, affecting estradiol and the rest of the hypothalamic axis. Graph 3.2 indicates that estradiol concentrations follow the same pattern between the two groups, and that the VNO has little effect on estradiol secretion or control which indicates normal ovarian function in both groups, even if the treatment group did not ovulate. In most mammals, including goats, estrogens are produced by the ovaries, but LH is produced by gonadotropic cells in the Pars distalis of the hypophysis (Bearden et al, 2004). However, LH secretion is controlled by GnRH which are secreted by the paracellular cells in the hypothalamus (Dellmann 1976). Since studies have shown that anatomical and biochemical pathways connect the VNO with the hypothalamus, impaired VNO function must therefore have a direct effect on GnRH secretions in goats (Quanbeck et al, 1997; Schwanzel-Fukuda and Pfaff, 1989; Wray et al, 1989; Yoshida et al, 1995; Yoshida et al, 1999). Graph 3.4 demonstrate in anoestrus does with a functional VNO, that stimulation of the VNO has a stimulatory effect on the secretion of GnRH, which causes an increase in the pulsatile release of GnRH and subsequently LH. This increase in secretion frequency synchronizes the secretion patterns of LH and estradiol and in turn increases the efficacy of the feedback mechanism with which these hormones are controlled. This stimulates follicular growth and ultimately ovulation. It is thus clear that the frequency of LH secretion and its synchronization with the secretion patterns of other reproductive hormones like estradiol and FSH is more important than the absolute concentration of any one of these hormones.

The data in the experiment showed that the typical effect of a male on a group of cyclic does is to synchronize oestrus, and the typical effect of a male on an anoestrus doe is to synchronize the secretion patterns of the hypothalamic axis, and so inducing a return to oestrus. The hypothalamus is also sensitive to the feedback exerted on it by FSH and estradiol. If the control exerted by the VNO was simply to increase the frequency of LH secretion, and the sensitivity of the hypothalamus to the feedback activities of estradiol and FSH remained constant, LH would not be expected to reach such high values in anoestrus does. High concentrations of LH in

the hypothalamus would immediately elicit a response from FSH which would exert a negative feedback effect onto GnRH secretion. However, this in itself would preserve the synchronized secretion pattern for LH, FSH and estradiol, thus preserving fertility. This investigation shows that stimulation of a functional VNO also effected the sensitivity of GnRH neurons on the negative feedback exerted by FSH and estradiol. It is very possible that the very same biochemical factors which stimulates the release of GnRH, can also affect receptor function and sensitivity.

Cortisol:

Reproduction is negatively influenced by stress and cortisol is secreted in response to stress (Bearden et al, 2004). Graphs 3.5 and 3.6 show that impaired VNO function had a definite effect on the stress as measured by cortisol secretion. This effect on cortisol could be a secondary effect due to difficulty in identifying other individuals, or it could be a more primary effect. This could be affected by the same or similar pathway that connects the VNO with the hypothalamus. In this case the secretion of corticotrophin releasing factor (CRH) could be either affected as a primary or secondary response to VNO stimulation. It is thus possible that the same stimuli that cause GnRH secretion could affect CRH release. The only way to substantiate the possibility of a direct vomeronasal influence on CRH release would be to measure CRH and adrenocorticotrophic hormone (ACTH) release in a similar study.

The results of this experiment also indicate that vomeronasal impairment had an effect on the secretion of cortisol, as is illustrated in Graphs 3.5 and 3.6. One possible explanation that can be presented is based upon the reported inability of ewes with non-functional VNO's to discriminate between their own lambs and foreign lambs (Booth and Katz, 2000). These ewes allowed all lambs to suckle, while those with functional VNO's continuously rejected strange offspring and only allowed their own offspring to suckle. If the VNO is responsible for pheromone detection, and animals identify each other through specific pheromones, then any disruption in VNO function should impair the ability of individuals to identify other individuals (Booth and Katz, 2000). Thus, when a strange male is introduced to a group of normal females with functional VNO's a short term increase in cortisol can be expected, however, it is expected that cortisol will normalize as the does identify the male. If a female does not have a functional VNO, and is therefore not able to identify another individual, a sustained increase in cortisol can be expected for a period after the first introduction. This would be exaggerated if a strange, sexually mature male is introduced to a group of females with non-functional VNO's. It would also be expected that cortisol will maintain a higher base level in vomerolectomized females than untreated females, as shown in Graph 3.6.

The mean cortisol values between the control and treatment groups in this study differed significantly ($P = 0.02$). Graph 3.6 indicates that male introduction in the treatment group (from T_4) had a definite effect on cortisol secretion. This increase was however short lived and cortisol stabilized again after a short period of time. However, in the control group, there is a trend toward a slight increase over time in $Cort_c$. This is due to the effect of the continuing blood sampling throughout the trial period had on the stress levels of the does.

Cortisol levels in both groups in this study followed much the same pattern, except for the following.

1. The initial levels of cortisol in the treatment group was higher than that of cortisol in the control group, indicating that the treatment does were already experiencing higher stress levels before the trial period began, despite being subjected to the same experimental conditions as the control group. This is due to the difficulty in identifying other individuals via a non-functional VNO, and interpreting and adapting to changes in the immediate environment.
2. After male introduction (T_3), $Cort_T$ continually increases throughout the trial period, indicating difficulty in identifying the newly introduced males.
3. $Cort_T$ and $Cort_c$ both peak at T_7 , with $Cort_T$ statistically higher than $Cort_c$ ($P=0.04$)
4. $Cort_T$ also declines sharply at T_7 - T_8 , but only to a level close to that of T_1 and T_2 (basal level). At T_9 $Cort_T$ stabilizes visibly at a higher level than $Cort_c$.

The treatment group had a different reaction to male introduction than the control group. Both groups were subjected to the same conditions and handling since the graphs show some similarities, however these similarities are overshadowed by the difference in blood levels of $Cort_c$ and $Cort_T$. The treatment does demonstrated higher blood levels of cortisol throughout the entire time with the males. This indicates that the treatment does experienced higher levels of stress during the trial period than control does. This indicates an inability to interpret and adapt to changes in their immediate environment, especially changes in the immediate herd composition. Although the control group was exposed to the same changes in their immediate environment, they were able to adapt to these changes as indicated by a more stable secretion pattern of cortisol, but also higher fertility as indicated by a higher conception rate.

Cortisol can be affected in a number of possible ways (Swenson and Reece, 1993):

1. CRH release is affected directly by a similar pathway that stimulates GnRH release from the hypothalamus.
2. CRH inhibition is a secondary effect to LH and estradiol secretion.

3. Cortisol and estradiol secretory patterns exhibit similarities because both hormones are synthesized by the same metabolic pathway.
4. CRH and cortisol secretion is independent from LH and estradiol secretion, but has a secondary effect on the reproductive axis.

The cortisol results can be explained by understanding the effect of cortisol on different tissues and systems, especially on the nervous system. Cortisol is a glucocorticoid and its main effect is on carbohydrate metabolism (Swenson and Reece, 1993). Swenson and Reece (1993) highlighted studies investigating adrenal insufficiencies indicating an effect on the central nervous system (CNS). It was found that electrical discharge in the central nervous system is lowered after adrenalectomy (Swenson and Reece, 1993). When cortisol is injected, electrical discharge is increased due to a lowering of the cellular threshold for electrical excitation. Another effect of cortisol insufficiency is increased sensitivity of olfactory and gustatory receptors (Swenson and Reece, 1993). However, vomeronasal receptors were not included in these studies.

Based on the above it is possible to construct a possible explanation for the increase in cortisol levels, as illustrated in Graph 3.6. The occlusion of the vomeronasal duct prevents the animal from identifying individuals in its immediate environment, however through the other senses the animal is aware that other individuals are present. The inability to identify other members of the herd was illustrated in the study by Booth and Katz (2000) and was also observed in this study. Booth and Katz (2000) also reported that vomerolectomized ewes that were presented with both their own and foreign lambs continued to sniff the tails of the presented lambs and performed “maternal flehman” in response, they also permitted all lambs to suckle. This behaviour continued while control ewes were able to immediately identify the lamb as her own, or as foreign. Consequently foreign lambs were rejected. This inability to identify herd members manifests itself as an increase in stress and thus an increase in cortisol secretion. This increase in cortisol secretion increases the sensitivity of related receptors in the nasal and oral epithelium, in an attempt to compensate for the loss of vomeronasal receptor function. This response in cortisol must however be interpreted as a secondary effect to stress. Cortisol secretion did not increase as a direct effect of VNO impairment, but as a secondary effect due to the increase in stress experienced by the treatment animals, since cortisol secretion did not decrease to levels similar to those of the control group. Cortisol is also not increased to affect the sensitivity of other receptors per se, but the effect on gustatory and olfactory receptors is part of the “permissive” effect of cortisol to prepare the animal to deal with stress (Swenson and Reece, 1993).

The data in this study demonstrates that male introduction (T_3) has an effect on Est_c , LH_c and on $Cort_c$. In the treatment group male introduction only had an effect on estradiol and cortisol concentrations, with no increase

in LH secretion during the pro-oestrus period (T_4 - T_6), as was demonstrated in the control group. Graph 3.6 however, shows a constant increase in cortisol over time in both groups, with a sudden decrease right at the end of the trial period.

Swenson and Reece (1993) also reports that excess glucocorticoids can affect gonadotropin secretion as a primary inhibitor or as a secondary inhibitor that increases hypophyseal and hypothalamic sensitivity to negative feedback by sex steroids. In the control group the classic relationship between the gonadotropins, sex steroids and glucocorticoids was observed. When the same relationships are compared to the treatment group, clear anomalies become apparent. $Cort_T$ and Est_T move in opposite directions, while LH_T shows an absence in LH surge at male introduction (T_3).

After 36 hours after male introduction, all of the does in the control group were serviced while none of the does in the treatment group were serviced. In fact, many of the does in the treatment group ran away from the bucks at first, showing that these does couldn't recognize the bucks at buck introduction and for many hours after.

Conclusion

The vomeronasal system plays an important role in the social interaction between bucks and does. This role not only extends into the socio-sexual behaviour, but also into the modulation of the reproductive system as a whole. This study shows that the vomeronasal system plays a more important role than simply identifying mating partners. It plays a crucial role in the preparation of not only the individuals for mating, but also the reproductive system to ensure successful reproduction. The vomeronasal system is crucial in linking mating behaviour with reproductive success, and linking reproductive processes with each other at the right time. A non-functional vomeronasal system not only upsets the delicate balance between the reproductive and adrenal axes, but also the mechanisms that maintain this delicate balance. The breakdown of these systems and their balancing mechanisms results in the uncoupling and de-synchronization of the normal series of events that is crucial to ensure successful ovulation and successful reproduction of the species. Furthermore it uncouples the physiological processes from the behavioural processes, which has detrimental effects on successful reproduction.

The data from this trial would suggest that the VNO exerts an effect on the reproductive system through two main routes:

1. The modulation of the secretion pattern and frequency of GnRH, and subsequently LH, to synchronize the secretion patterns of LH, FSH and estradiol. This synchronization precipitates follicular development and ultimately ovulation.
2. The VNO increases the sensitivity of the hypothalamus to the feedback effects of FSH and estradiol. This assists in the synchronization of the secretion patterns of the relevant hormones involved.

One of the most important factors in determining the success of a small stock operation is the rate of reproduction. Reproductive efficiency is influenced by a number of variables, including environment, nutrition and genetics. Behaviour is also an important aspect of reproduction, and an understanding of the basics of reproductive behaviour can lead to management applications that can improve reproductive success. Reproduction leads to profit and it is thus helpful to understand the effects that the different sexes of goats have on each other so that one can exploit the most of the “male effect” to have a successful farming enterprise. The advantages of the “male effect” are that it does not require external administered hormones, it advances the breeding season by a few weeks and it synchronizes ewes so that the lambing period is much shorter (Rosa and Bryant, 2002).

The result of this study indicates that in goats, the VNO is needed for reproduction as none of the does in the treatment group fell pregnant and all of the does in the control group fell pregnant. This study also demonstrated that increased levels of LH, GnRH, estradiol and other hormones are needed for female animals to go into and show heat, and this is also regulated or modulated by a functional VNO. Due to the increased cortisol levels (increased stress), this study also indicates that a functional VNO is needed for animals to identify each other in groups or in a herd.

Bibliography

1. Abass, T.A., Al-Mayahi, M.S., Al-Hussany, B.F., 2012. Anatomical and histological investigate of vomeronasal organ (VNO) in Iraqi sheep Alawasi. *Kufa journal for veterinary medical sciences*, 3(1):98-107.
2. Abbot, D.H. and Hearn, J.P., 1978. Physical, hormonal and behavioral aspects of sexual development in the marmoset monkey, *Callithrix jacchus*. *Journal for Reproduction and Fertility*, 53:155-166.
3. Abecia, J.A., Forcada, F. and Zuñiga, O., 2002. A note on the effect of individual housing conditions on LH secretion in ewes after exposure to a ram. *Applied Animal Behaviour Science* 75:347-352.
4. Avdi, M., Leboeuf, B. and Terqui, M., 2004. Advanced breeding and “buck effect” in indigenous Greek goats. *Livestock Production Science*, 87:251-257.
5. Bailey, K., 1977. Flehman in the Ring tailed Lemur. *Behaviour* LXV, 3-4:309-319.
6. Bearden, H.J., Fuquay, J.W. and Willard, S.T., 2004. *Applied animal reproduction*, 6th edition. Pearson Prentice Hall, New Jersey: 2004.
7. Błaszczuk, B., Udała, J. and Gączarzewics, D., 2004. Changes in estradiol, progesterone, melatonin, prolactin and thyroxine concentrations in blood plasma of goats following induced estrus in and outside the natural breeding season. *Journal for Small Ruminant Research*, 51:209-219.
8. Booth, K.K. and Katz, L.S., 2000. Role of the vomeronasal organ in neonatal offspring recognition in sheep. *Biology of Reproduction*, 63:953-958.
9. Booth, K.K., and Webb, E.C., 2011. Effect of blockage of the ducts of the vomeronasal organ on the LH plasma levels during the “whitten effect” in does. *SAGE – Hindawi Access to Research, Veterinary Medicine International*, Volume 2001.
10. Bouret, S., Prevot, V., Croix, D., Viltart, O., Stefano, G.B., Mitchell, V. and Beauvillain, J., 2000. Opioid receptor mRNA expression in neuronal nitric oxide synthase-immunopositive preoptic area neurons. *Molecular Brain Research*, Volume 80, 1:46-52.
11. Breed, W.G., 1976. Effect of environment on ovarian activity of wild hopping mice (*Notomys alexis*). *Journal for Reproduction and Fertility*, 47:395-397.
12. Broadwell, R.D., 1975. Olfactory relationships of the telencephalon and diencephalons in the rabbit. *Journal of Comparative Neurology* 163:329-346.
13. Brooks, P.H. and Cole, D.J.A., 1970. Effect of the presence of a boar on attainment of puberty in gilts. *Journal for Reproduction and Fertility*, 23:435-440.

14. Bronson, F.H., 1982. Pheromonal influences on endocrine regulation of reproduction; Endocrine responses to primer pheromones in mammals: Olfaction and endocrine Regulation (ed. W. Breipohl). IRL Press Limited. Pp 103-113.
15. Bruce, H., 1960. A block to pregnancy in the mouse caused by the proximity of strange males. *Journal for Reproduction and Fertility*, 1:96–103.
16. Castro, B.M., 1967. Age of puberty in female mice: relationship to population density and the presence of adult males. *Anais Academia Brasileira de Ciencias*, 39:289-291.
17. Chemineau P, Levy F, Thimonier J., 1983. Effects of anosmia on LH secretion, ovulation and oestrous behaviour induced by males in the anovular Creole goat. *Journal of Animal Reproduction Science*. 1986; 10(2):125–132.
18. Clarke, I.J., Scott, C.J., Pereira, A and Pompolo, S., 2005. The role of noradrenaline in the generation of the preovulatory LH surge in the ewe. *Journal of Domestic Animal Endocrinology*, 30(4):260-275.
19. Cohen-Tannoudji, J., Lavenet, C., Locatelli, A., Tillet, Y., and Signoret, J.P., 1989. Non-involvement of the accessory olfactory system in the LH response of anoestrus ewes to male odour. *Journal of Reproduction and Fertility*, 86:135-144.
20. Crowcroft, P. and Rowe, F.P., 1957. The growth of confined colonies of the wild house-mouse (*Mus musculus L.*). *Journal of Proceedings of the Zoological Society of London*, 192:359-370.
21. Dagg, A.I. and Taub, A., 1970. Flehman. *Journal for Mammalia*, 34:686-659.
22. Davis, B.J., Macrides, F., Youngs, W.M., Schneider, S.P. and Rosene, D.L., 1977. Efferents and Centrifugal Afferents of the Main and Accessory Olfactory Bulbs in the Hamster. *Brain Research Bulletin*, 3:59-72.
23. Dees, W.L., Dearth, R.K., Hooper, R.N., Brinsko, S.P., Romano, J.E., Rahe, H., Yu, W.H. and McCann, S.M., 2001. Lamprey gonadotrophin-releasing hormone-III selectively releases stimulating hormone in the bovine. *Domestic Animal Endocrinology*, 20(4):279-288.
24. Dellmann, H.D., 1976. Endocrine system in *Textbook of Veterinary Histology*, pp 373-399. Lea and Febriger, Philadelphia, Pa, USA.
25. Dominic, C.J. and Pandey, S.D., 1979. Suppression of oestrus-inducing pheromone production in the Indian field mouse, *Mus booduga Gray*, by treatment with an anti-androgen. *Journal of Endocrinologie*, 74:1-5.
26. Doving, K.B. and Trotier, D., 1998. Structure and function of the vomeronasal organ. *Journal of Experimental Biology*, 201:2913-2925.
27. Drickamer, L.C., 1974. Sexual maturation of female house mice: social inhibition. *Journal of Developmental Psychobiology*, 7:257-265.

28. Drickamer, L.C. and Hoover, J.E., 1979. Effects of urine from pregnant and lactating female house mice on oestrus cycle of adult females. *Journal of Developmental Psychobiology*, 12:545-551.
29. Drickamer, L.C. and Murphy, R.X., 1978. Female mouse maturation: effects of extreted and bladder urine from juvenile and adult males. *Journal of Developmental Psychobiology*, 11:63-72.
30. Drickamer, L.C., McIntosh, T.K. and Rose, E.A., 1978. Effects of ovariectomy on the presence of a maturation-delaying pheromone in the urine of female mice. *Journal of Hormonal behaviour*, 11:131-137.
31. Dulac, C., 2000. Sensory Coding of pheromone signals in mammals. *Journal of Current Opinion in Neurobiology*, 10:511-518.
32. Dulac, C. and Axel, R., 1995. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* 83: 195-206.
33. Estes, R.D., 1972. The role of the vomeronasal organ in mammalian reproduction. *Journal of Mammalia*, 36(3):315-341.
34. Faruk, M.I., Fatema, B.Z., Bari, F.Y., and Alam, M.G.S., 2004. Effects of alfaprostol and luprostirol on the oestrus synchronization response in black Bengal goats. *Bangladesh Journal of Veterinary Medicine*, 2(2):143-145.
35. Fewell, G.D. and Meredith, M., 2002. Experience facilitates vomeronasal and olfactory influence on Fos expression in medial preoptic area during pheromone exposure or mating in male hamsters. *Journal of Brain Research*, 941(1-2):91-106.
36. Graham, C.A. and McGrew, W.C., 1980. Menstrual synchrony in female undergraduates living on a co-educational campus. *Journal of Psychoneuroendocrinology*, 5:245-252.
37. Halpern, M. and Martinez-Marcos, A., 2003. Structure and function of the vomeronasal system: an update. *Progress in Neurobiology* 70(3):245-318.
38. Hamadeh, S.K., Abi Said, M., Tami, F. and Barbour, E.K., 2001. Weaning and the ram effect on fertility, serum luteinizing hormone and prolactin levels in spring rebreeding of port partum Awassi ewes. *Journal of Small Ruminant Research* 41:191-194.
39. Handelmann, G., Ravizza, R. and Ray, W.J., 1980. Social dominance determines estrous entrainment among female hamsters. *Journal of Hormonal behaviour*, 14:107-115.
40. Izard, M.K., 1983. In "Pheromones and Reproduction in mammals" (Vandenburgh, ed) Academic Press incorporated, New York, 253-285.
41. Izard, M.K. and Vandenbergh, J.G., 1982. The effects of bull urine on puberty and calving date in crossbred beef heifers. *Journal of Animal Science*, 55:1160-1168.

42. Jia, C. and Halpern, M., 1996. Subclasses of vomeronasal receptor neurons: differential expression of G proteins ($G_{i\alpha 2}$ and $G_{o\alpha}$) and segregated projections to the accessory olfactory bulb. *Journal of Brain Research*, 719(1-2):117-128.
43. Johns, M.A., Feder, H.H., Komisaruk, B.R. and Mayer, A.D., 1978. Urine-induced reflex ovulation in anovulatory rats may be a vomeronasal effect. *Nature*, 272(5652): 446-448.
44. Karlson, P. and Butenandt, A., 1959. Pheromones (Ectohormones) in insects. *Journal of Annual Reviews, Entomology*, 4:39-58.
45. Kappers, C.U.A., Huber, G.C. and Crosby, E.C., 1936. The comparative anatomy of the nervous system of vertebrates, including man. The Macmillan Co., New York.
46. Kasikci, G., Cirit, U., Gunduz, M.C., Bacimoglu, S., 2011. Effects of halving intravaginal sponges and sCG dose on estrus response and fertility in Tahirova ewes during the breeding season. *Turkish Journal of Veterinary Animal Science*, 35(3):193-199.
47. Katz, Y and Epplé, G., 1980. Social influences on first reproductive success and related behaviors in the saddle-back Tamarin (*Saguinus fuscicollis*). *Journal of Primatology*, 1:171-183.
48. Keller, M., Bedos, M., Jouhanneau, M., Martini, M., Ratana-Marquez, S., and Delgadillo, J.S., 2012. Pheromonal modulation of reproductive function in mammals. *Journal of Tropical and Subtropical Agroecosystems*, 15:S103-S111.
49. Keverne, E.B., 1983. Pheromonal influences on the endocrine regulation of reproduction. *Trends in Neuroscience*, 6:381-384.
50. Keverne, E.B., Murphy, C.L., Silver, W.L., Wysocki, C.J., and Meredith, M., 1986. Non-olfactory chemoreceptors of the nose: recent advances in understanding the vomeronasal and trigeminal systems. *Journal of Chemical senses*, 11:119-133.
51. Keverne, E.B., 2004. Importance of olfactory and vomeronasal systems for male sexual function. *Journal of Physiology and Behaviour*, 83:177-187.
52. Keveter, G.A. and Winans, S.S., 1981. Connections of the Corticomedial Amygdala in the Golden Hamster: I. Efferents of the "Vomeronasal Amygdala". *Journal of Comparative Neurology*, 197:81-98.
53. Knappe, H., 1964. Zur funktion des Jacobsonschen Organs (Organon vomeronasale Jacobsoni). *Zoology Gartem (NF)*, 28:188-194.
54. Knights, M., Baptiste, Q.S. and Lewis, P.E., 2002. Ability of ram introduction to induce LH secretion, estrus and ovulation in fall-born ewe lambs during anestrus. *Journal of Animal Reproduction Science*, 69:199-209.

55. Krieger, J., Schmitt, A., Löbel, D., Gudermann, T., Schultz, G., Breer, H. and Boekhoff, I., 1999. Selective activation of G-protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds. *Journal of Biological Chemistry*, 274:4655-4662.
56. Ladewig, J., and Hart, B.L., 1980. Flehman and vomeronasal organ function in male goats. *Journal of Physiology and Behaviour*, 24:1067-1071.
57. Lawton, A.D. and Whitsett, J.M., 1979. Inhibition of sexual maturation by a urinary pheromone in male prairie deer mice. *Hormone behaviour* 13:128-138.
58. Liman, E.R., 2001. Sex and the single Neuron: Pheromones excite. *Trends in Neuroscience*, 24(1):2-3.
59. Lohman, A.H.M. and Mentink, G.M., 1969. The lateral olfactory tract, the anterior commissure and the cells of the olfactory bulb. *Journal of Brain Research*, 12: 396-413.
60. Lombardo, D.L. and Terman, C.R., 1980. The influence of the social environment on sexual maturation of female prairie deermice *Peromyscus maniculatus bairdii*. *Journal of Research Population Ecology*, 22:93-100.
61. Martin, G.B., Milton, J.T.B., Davidson, R.H., Banchemo Hunzicker, G.E., Lindsay, D.R. and Blache, D., 2004. Natural methods for increasing reproductive efficiency in small ruminants. *Journal of Animal Production Science*, 82-83:231-246.
62. Martin, G.B., Oldham, C.M., Cognié, Y. and Pearce, D.T., (1986) The physiological responses of anovulatory ewes to the introduction of rams—a review. *Journal of Livestock Production Science*, 15:219–247.
63. McClintock, M.K., 1971. Menstrual synchrony and suppression. *Journal of Nature*, 229:244-245.
64. McClintock, M.K., 1978. Estrous synchrony and its mediation by airborne chemical communication. *Journal for Hormonal behaviour*, 10:264-276.
65. McClintock, M.J., 1983. Pheromonal Regulation of the Ovarian Cycle: Pheromones and Reproduction in Mammals (ed. J.G. Vandenbergh), Academic Press, Inc, New York, 113-149.
66. McIntosh, T.K. and Drickamer, L.C., 1977. Excreted urine, bladder urine, and the delay of sexual maturation in female house mice. *Journal of Animal Behaviour*, 25:999-1004.
67. Meredith, M., 1983. In “Pheromones and Reproduction in Mammals” (J.G. Vandenburgh, ed). Academic Press, New York, 199-252.
68. Over, R., Cohen-Tannoudji, J., Dehnhard, M., Claus, R., and Signoret, J.P., 1990. Effect of pheromones from male goats on LH-secretion in anoestrus ewes. *Journal of Physiology and behaviour*, 48:665-668.
69. Pantages, E. and Dulac, C., 2000. A novel family of candidate pheromone receptors in mammals. *Neuron Journal*, 28:835-845.

70. Patterson, D.J., Kojima, F.N., Stegner, J.E., Bader, J.F., Perry, G.A., Schafer, D.J. and Smith, M.F., 2004. Review of estrus synchronization systems:MGA. Proceedings, Applied Reproductive strategies in Beef Cattle, University of Missouri, Columbia, North Platte, Nebraska.
71. Payman, B.C. and Swanson, H.H., 1980. Social influences on sexual maturation and breeding in the female Mongolian gerbil *Meriones unguiculatus*. *Journal of Animal Behaviour*, 28:528-535.
72. Quadagno, D.M., Shubeita, H.E., Deck, J. and Francoeur, D., 1981. Influence of male social contacts, exercise and all female living conditions on the menstrual cycle. *Journal of Psychoneuroendocrinology*, 6:239-244.
73. Quanbeck, C., Sherwood, N.M., Millar, R.P. and Terasawa, E., 1997. Two populations of luteinizing hormone-releasing hormone neurons in the forebrain of the rhesus macaque during embryonic development. *Journal of Comparative Neurology*, 380(3):293-309.
74. Raisman, G., 1972. An Experimental Study of the Projection of the Amygdala to the Accessory Olfactory Bulb and its Relationship to the Concept of a Dual Olfactory System. *Journal for Experimental Brain Research*, 14:395-408.
75. Reynolds, J. and Keverne, E.B., 1979. Accessory olfactory system and its role in pheromonally mediated suppression of oestrous in grouped mice. *Journal of Reproduction and Fertility*, 57:31-35.
76. Rosa, H.J.D. and Bryant, M.J., 2002. The "ram effect" as a way of modifying the reproductive activity in the ewe. *Journal of Small Ruminant Research*, 45:1-16.
77. Sanchez-Criado, J.E., 1982. Involvement of the vomeronasal system in the reproductive physiology of the rat: *Olfaction and Endocrine Regulation* (ed. W. Breiphol), IRL Press Limited, London, 209-221.
78. Scalia, F. and Winans, S.S., 1975. The differential Projections of the Olfactory Bulb and the Accessory Olfactory Bulb in Mammals. *Journal of Comparative Neurology* 161:31-56.
79. Scwanzel-Fukuda, M. and Pfaff, D.W., 1989. Origin of luteinizing hormone releasing hormone neurons. *Nature*, 338:161-164.
80. Signoret, J.P. and Lindsay, D.R., 1982. The male effect in domestic mammals: effect on LH secretion and ovulation. *Importance of olfactory cues. Olfaction and Endocrine Regulation* (ed. W. Breipohl). IRL Press, London, 63-72.
81. Stabenfeldt, G.H. and Edqvist, L., 1993. Female reproductive processes. *Duke's Physiology of Domestic Animals* (Swenson, M.J. and Reece, W.O. ed.), 692-693.
82. Stoddard, D.M., 1976. Mammalian odours and pheromones. *The Institute of Biology's Studies in Biology*, 73:1-14.
83. Swenson, M. J. and Reece, W. O., 1993. *Duke's Physiology of Domestic Animals* 11th Edition. Comstock publishing associates a division of Cornell University press, USA.

84. Takami, S., 2002. Recent progress in the neurobiology of the vomeronasal organ. *Journal of Microscopy Research and Technique*, 58(3):228-250.
85. Ungerfeld, R., 2003. Reproductive responses of anestrus ewes to the introduction of rams. Doctoral thesis at the Swedish University of Agricultural Sciences, Uppsala, Sweden.
86. Ungerfeld, R., Suárez, G., Carbajal, B., Silva, L., Laca, M., Forsberg, M. and Rubianes, E., 2003. Medroxyprogesterone priming and response to the ram effect in Corriedale ewes during the nonbreeding season. *Journal of Theriogenology*, 60(1):35-45.
87. Vandenberg, J.G., 1967. Male odor accelerates female sexual maturation in mice. *Journal of Endocrinology*, 84:658.
88. Vandenberg, J.G., 1971. The influence of the social environment on sexual maturation in male mice. *Journal of Reproduction and Fertility*, 24:383-390.
89. Vandenberg, J.G., 1983. Pheromonal regulation of puberty: Pheromones and Reproduction of Mammals (ed. J.G. Vandenberg), Academic Press, Inc, New York, 95-112.
90. Whitsett, J.M., Noden, P.F., Cherry, J and Lawton, A.D., 1984. Effect of transitional photoperiods on testicular development and puberty in male deer mice (*Peromyscus maniculatus*). *Journal of Reproduction and Fertility*, 72:277-286.
91. Whitten, W.K., 1985. Vomeronasal organ and the accessory olfactory system. *Applied Animal Behaviour Science*, 14:105-109.
92. Wilson, E.O. and Bossert, W.H., 1963. Chemical communication among animals. *Journal of Recent Progress in Hormone Research*, 19:673-710.
93. Winans, S.S., Lehman, M.N. and Powers, J.B., 1982. In "Olfaction and Endocrine Regulation" (W. Breipohl, ed). IRL Press Ltd, London, 23-34.
94. Winfree, A.T., 1980. *The Geometry of Biological Time*. Springer-Verlag, Berlin & New York.
95. Wray, S., Nieburgs, A. and Elkabes, S., 1989. Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. *Developmental Brain Research*, 46(2):309-318.
96. Wysocki, C.J., and Wellington, J.L., 1980. Access of urinary non-volatiles to the mammalian vomeronasal organ. *Journal of Science*, 207:718-783.
97. Wysocki, C.J., Beauchamp, G.K., Reidinger, R.R. and Wellington, J.L., 1985. Access of large and non-volatile molecules to the vomeronasal organ of mammals during social and feeding behaviors. *Journal of Chemical Ecology*, 11(9):1147-1159.

98. Yoshida, K., Tobet, S.A., Crandall, J.E., Jimenez, T.P. and Schwarting, G.A., 1995. The migration of luteinizing hormone-releasing hormone neurons in the developing rat is associated with a transient, caudal projection of the vomeronasal nerve. *Journal of Neuroscience*, 15(12):7769-7777.
99. Yoshida, K., Rutishauser, U., Crandall, J.E. and Schwarting, G.A., 1999. Polysialic acid facilitates migration of luteinizing hormone-releasing hormone neurons on vomeronasal axons. *Journal of Neuroscience*, 19(2):794-801.
100. Zufall, F., Leinders-Zufall, T. and Puche, A.C., 2008. *The senses: A comprehensive reference*, 4:783-814.