Studies into the effects of gonadectomy on the canine urinary bladder with reference to acquired urinary incontinence in the bitch

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Abstract

Acquired urinary incontinence in the canine is a distressing and debilitating condition affecting up to 20% of neutered bitches, whilst less than 1% of entire bitches and males suffer from this condition. Although a number of medical and surgical therapies exist for treatment of acquired urinary incontinence, none are able to cure the condition and many animals become refractory to treatment over time. It has long been thought that a decrease in resting tone within the urethra of a bitch following neutering is responsible for the development of acquired urinary incontinence; however, recent studies show that low urethral tone does not always lead to urinary incontinence, suggesting further factors must be involved. Although the exact aetiology and pathophysiology of the condition in the neutered bitch is unknown, it is thought to have many similarities to that of post menopausal urinary incontinence in women. In this condition, urinary incontinence is known to be mediated primarily by changes in the structure and function of the urinary bladder post menopause. The present study looks at the structure and function of the canine urinary bladder *in vitro* to determine if changes occur post neutering that could lead a bitch to develop acquired urinary incontinence and which may provide novel therapeutic targets for treatment of this disease.

Contractility in response to carbachol (muscarinic) and electrical field stimulation was assessed in isolated strips of detrusor muscle from male and female, intact and gonadectomised canines. The potential role of non-adrenergic, non-cholinergic mediated contraction of the detrusor muscle was also examined and this system does not appear to be significantly altered by gonadectomy. Maximal contractile responses were, however, decreased in detrusor strips from neutered compared to entire canines regardless of gender, with detrusor strips from incontinent bitches having some of the weakest responses. Sensitivity to carbachol was also decreased in detrusor strips from neutered compared to entire canines. This suggests a decrease in contractile function of the urinary bladder in neutered canines and is similar to that seen in the bladders of women suffering from urinary incontinence post-menopause due to impaired contractility of the bladder and idiopathic detrusor instability. This suggests that changes in the function of the bladder post neutering may be partly responsible for the development of acquired urinary incontinence in the bitch.

Post-menopausal urinary incontinence in women is hypothesised to be a linked to an increase in the collagen to smooth muscle ratio within the wall of the urinary bladder

which is thought to impair bladder contractility and lead to the development of detrusor instability. Morphometric analysis of the urinary bladder wall of canines showed that the percentage of collagen within this organ was significantly increased in neutered compared to entire bitches, with incontinent bitches having some of the highest percentage collagen. The percentage of collagen was unchanged in neutered compared to entire males which were similar to entire bitches. These results support the long postulated theory that a decrease in oestrogen following gonadectomy / menopause is involved in the increase of collagen within the bladder.

Results describing the pharmacological characterisation of muscarinic receptors (Schild analysis of pK_B values) in strips of canine detrusor muscle suggest that the M_3 receptor is the primary receptor responsible for bladder contraction in entire canines *in vitro* but that the M_2 receptor predominates in neutered canines. This previously unreported finding could be significant in providing a novel therapeutic target to treat this debilitating disease.

Studies that looked at mRNA expression for the muscarinic as well as the LH and GnRH receptors in canine bladder wall showed that there was an increase in expression of all receptors in tissue from neutered compared to entire canines and that tissue from females had higher expression levels than that from their male counterparts. It is known that gonadotrophin levels in the blood increase post neutering, and that decreasing these levels can provide continence in a number of animals. It is therefore possible, that an up-regulation of mRNA expression for these receptors is involved in the changes at the level of the detrusor that could lead to development of acquired urinary incontinence. It is also acknowledged that the muscarinic pathway is the primary pathway responsible for bladder contraction and emptying, therefore, a change in the expression of muscarinic receptors has the potential to alter bladder contractility as demonstrated previously.

In conclusion these studies have shown that the structure and function of the urinary bladder of a neutered canine is altered compared to that of an entire canine, and that these changes have the potential to be involved in the development of acquired urinary incontinence in the bitch. Many of these changes mimic those seen in the bladders of post menopausal women suffering from urinary incontinence, thus suggesting that there may be commonality of disease process between the two species which may allow the use of the canine as a model of human urinary incontinence. This data, the first to include male animals in the study of bladder function and structure, suggest that the loss of oestrogen in the female and the concurrent increase in percentage collagen within the urinary bladder are not significant factors in the development of decreased detrusor contractility per se. On

the contrary these results suggest that the muscarinic receptor effector pathway may play a crucial role in the development of altered bladder contractility and acquired urinary incontinence, and may provide a therapeutic target for effective treatment of this disease.

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Author's declaration

This dissertation is not substantially the same as any other I have submitted for a degree, diploma or any other qualification at any other institution. It is the result of my own work and includes nothing that is the outcome of work done in collaboration with others, except where stated.

Victoria Ann Coit

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Abbreviations

ACh	Acetylcholine
ATP	Adenosine triphosphate
AUI	Acquired urinary incontinence
cAMP	Cyclic adenosine-3',5'-monophosphate
CI	Confidence interval
CNS	Central nervous system
DAG	Diacylglycerol
4-DAMP	4-diphenyl-acetoxy-*N*-methyl piperidine methiodide
DO	Detrusor overactivity
FE	Entire female canine(s)
FN	Neutered female canine(s)
FN AUI	Neutered female canine(s) known to be suffering from acquired urinary incontinence
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
IDI	Idiopathic detrusor instability
IP_3	Inositol triphosphate
KCl	Potassium Chloride
LH	Luteinising hormone
ME	Entire male canine(s)
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase
MN	Neutered male canine(s)
PNS	Parasympathetic nervous system
SNS	Sympathetic nervous system
Ttx	Tetrodotoxin
UI	Urinary incontinence

1 Introduction

1.1 Overview of Urinary Incontinence

Urinary incontinence is defined as the involuntary and unconscious loss of urine from a patient, and can occur at anytime whether the patient is awake (enuresis) or sleeping (nocturnal enuresis) (Abrams *et al.*, 2002). Although there can be many varied causes of urinary incontinence including ectopic ureters, neoplasia of the bladder and / or urethra, trauma of the spinal cord, inflammatory diseases and physiological disorders (Table 1-1) there are only two populations known to be at significant risk of developing urinary incontinence: women and bitches. In these populations the majority of urinary incontinence is thought to be due to hormonal and / or age related changes. In women the risk of developing urinary incontinence increases after the menopause and in the bitch, after neutering (Abrams *et al.*, 2002; Holt *et al.*, 1993; Thom *et al.*, 1998; Thrusfield, 1985). Urinary incontinence is thought to have a multimodal aetiology and pathogenesis. It has been more closely defined and subcategorised in women and in this population falls under two main syndromes; urge incontinence, where the bladder contracts out-with conscious and voluntary patient control and stress incontinence where the tone within the urethral sphincters is insufficient to overcome any increases in pressure within the bladder, such as when sneezing occurs (Abrams *et al.*, 2002). It is recognised that many women suffer concurrently from these syndromes which can act in synergy with each other to cause an increase in the frequency or severity of the condition; this is defined as mixed urinary incontinence (Abrams *et al.*, 2002). In the canine only one form of urinary incontinence has been widely reported, variously known as post-neutering urinary incontinence, urethral sphincter mechanism incompetence and acquired urinary incontinence, the latter of which will be used in this thesis. Acquired urinary incontinence has mainly been likened to stress incontinence in women, whereby overt leakage of urine occurs mainly when the bitch is lying down or excited and actively moving (Arnold *et al.*, 1989; Hotston Moore, 2001). Despite this, some owners report that their bitch appears agitated just before an episode of urinary incontinence or inappropriate urination and that some bitches will try and get to their usual toileting place immediately before or during an episode, suggesting that some bitches may suffer from an undiagnosed form of urge incontinence.

Type	Example
Congenital	Ectopic ureter(s)
	Patent urachus
Gynaecological	Pregnancy and parturition
	Atrophy / pelvic floor muscle weakness
	Bladder outflow obstruction
Urological	Detrusor overactivity
	Urethral incompetence
	Urinary tract infections
	Bladder calculi
	Neoplasia of urinary tract
Medical	Diabetes
	Chronic Renal Failure
	Hypothyroidism
Functional	Arthritis (restricted mobility)
	Post-surgical immobility
Iatrogenic	Ovariohysterectomy / ovariectomy
	Drugs (e.g. steroids, tricyclic antidepressants)
	Complication of vaginal / urethra surgery
Neurogenic	Spinal cord lesion
	Diabetic neuropathy
Psychogenic	Dementia

Table 1-1. Common causes of urinary incontinence in bitches and women.

1.2 Prevalence of Urinary Incontinence

Urinary incontinence is a widespread problem in both bitches and women, although the exact prevalence of the condition is likely to be underestimated due to the fact that many patients and owners fail to report the problem (Knight-Klimas, 2004). This failure to report the condition is likely due to a number of reasons including embarrassment, lack of knowledge of urinary incontinence, low expectation of treatment options and the erroneous thinking that it is a normal part of aging. Currently urinary incontinence is estimated to affect between 10 and 40% of women (Parsons *et al.*, 2003), however this figure rises to 50 to 70% in institutionalised elderly patients (Ouslander *et al.*, 1982) where self reporting is not required. The prevalence of urinary incontinence in women increases with age (Parsons *et al.*, 2003), obesity (Dwyer *et al.*, 1988; Han *et al.*, 2006), parturition and hysterectomy (Thom *et al.*, 1997), the menopause (Hunskaar *et al.*, 2000) and is affected by race (Graham *et al.*, 2001). Similar factors also predispose bitches to acquired urinary incontinence as increasing ideal adult body weight, obesity and breed all contribute to the predisposition to urinary incontinence (Arnold *et al.*, 1989). There is a strong correlation between increasing body weight and increasing risk of developing urinary incontinence, with bitches weighing greater than 20kg having a 30% risk and bitches weighing less than 10kg having a 10% risk for developing urinary incontinence (Arnold *et al.*, 1989; Holt *et al.*, 1993). It is also reported that in the bitch there is a direct relationship between tail docking and neutering, with bitches that are docked having a higher incidence of urinary incontinence then their full tailed counterparts, possibly due to neurological changes brought about by trauma to the nerve roots as they leave the spinal canal (Holt *et al.*, 1993). This latter fact should play a diminishing role in the disease prevalence in the UK over the coming years as tail docking has now been effectively banned in all breeds for cosmetic purposes. As the breeds reported to be most affected by acquired urinary incontinence in the only published UK study of the disease prevalence are traditionally docked breeds (rottweiler, dobermann pinscher and boxer) (Holt *et al.*, 1993) it remains to be seen if the breed distribution of predisposition to acquired urinary incontinence will change over the coming years. Although the development of urinary incontinence can also be linked to mechanical damage of the urinary tract, following a difficult parturition or accidental trauma, this is only true for a minority of bitches. In the bitch the largest contributing factor to the development of acquired urinary incontinence is neutering, with up to 20% of neutered bitches (Arnold *et al.*, 1989), compared to <1% of intact bitches (Holt *et al.*, 1993) subsequently reported to develop acquired urinary incontinence. A direct relationship between neutering and acquired urinary incontinence has been reported

(Thrusfield, 1985) which is proposed to occur as a consequence of hormonal, vascular or neurological changes (Thrusfield *et al.*, 1998), rather than mechanical damage of the lower urinary tract sustained during surgery (Gregory, 1994).

Due to the relationship between neutering and the development of acquired urinary incontinence in bitches, there has been debate as to the best time to neuter a non-breeding bitch, be it before or after her first season. Traditionally it has been recommended to neuter a bitch before her first season as this is known to lower the risk of her developing mammary tumours in later life (Dorn *et al.*, 1968); conversely it was thought that this may increase the risk of her developing acquired urinary incontinence (Thrusfield *et al.*, 1998). A study at the start of this century concluded that bitches neutered before their first oestrus may actually be at lower risk of developing acquired urinary incontinence than those neutered after their first oestrus (Stocklin-Gautschi *et al.*, 2001). The same study also concluded that there was no difference in the prevalence of development of acquired urinary incontinence in bitches neutered via ovariohysterectomy, the mainstay of neutering in Britain, and ovariectomy, the procedure of choice in mainland Europe. This latter finding lends weight to the hypothesis that it is the removal of the ovaries and the subsequent hormonal changes that occur that is responsible for the development of acquired urinary incontinence in the bitch and not the surgery per-se, unlike in the woman where hysterectomy is a recognised risk factor in the development of urinary incontinence (Thom *et al.*, 1997).

1.3 Clinical Signs and Complications Associated with Urinary Incontinence

Regardless of the aetiology of urinary incontinence the clinical signs of overt leakage of urine are the same. The condition in both humans and canines is debilitating, causes many social and economical problems and has a severe negative impact on the health of affected individuals and their quality of life (Hotston Moore, 2001; Kelleher *et al.*, 1997). The majority of women that suffer from urinary incontinence alter their lifestyle in some way and employ incontinence pads to aid in management of their condition which can lead to a decrease in their self confidence and severely curtail the activities that they partake in, decreasing their quality of life (Getliffe *et al.*, 2007; Miller *et al.*, 2003). In some communities to suffer from urinary incontinence is to become a social outcast and, in parts of the third world, women are thrown out of their families and communities if it is found that they suffer from the condition. In canines the social stigmatism seen in humans that accompanies urinary incontinence does not affect them but may be directed against their owners and can cause significant mental anguish for those involved. This is often due to the fact that the use of incontinence pads is unsuitable in most bitches and thus animals frequently contaminate their bedding, as well their environment, with urine. This environmental contamination is a potential health threat, as well as being socially unacceptable to many owners and may lead to alterations in the management of the affected canine and on occasions to their euthanasia. The management and lifestyle alterations that are undertaken by patients or the owners of bitches suffering from urinary incontinence are often costly both in terms of quality of life and economically and it is this latter issue which puts a large financial strain on individuals and in the case of women, the health service provider (Klotz *et al.*, 2007; Wilson *et al.*, 2001).

As well as the economic and social impact of urinary incontinence there are many ethical and welfare concerns for those affected (Hotston Moore, 2001). The constant leakage of urine can lead to scalding of the skin causing significant irritation and discomfort to the patient, potentially leading to secondary infections of the skin which, if left untreated, can become life-threatening (Holt, 1983; Kelleher, 2001). Along with the risk of skin infections there is also an increased incidence of cystitis among urinary incontinent patients, due to retrograde infections, which can progress to pylonephritis and kidney damage over time (Kelleher, 2001). In women, it is possible for prolapse of the vagina, bladder and urethra to occur in incontinent patients which is a severe condition usually requiring surgery to rectify; this complication has not been reported in the bitch. Urinary

incontinence also has a psychological impact on the patient and those caring for them, and can lead to severe psychosomatic problems such as anxiety, stress related disorders and depression (Miner, 2004; Zorn *et al.*, 1999).

1.4 Urodynamic Categorisation of Urinary Incontinence

The mechanisms that underlie urinary incontinence are more defined in women than in canines, as indicated earlier, due to the relative ease of urodynamic studies and the following subclassification of the condition in this species. The characteristics and known urodynamic causes of urinary incontinence in women and canines are reviewed below.

1.4.1 In the Woman

As previously stated, in women there are three main classifications of urinary incontinence; urge incontinence, stress incontinence, and mixed incontinence (Fig. 1-1). These classifications are based on the symptoms experienced by the patient and although these can be linked to possible mechanisms of disease do not necessarily relate to the underlying cause(s) of the disease. Urge urinary incontinence is the complaint of involuntary leakage of urine accompanied by or immediately preceded by urgency or the strong desire to void (Abrams *et al.*, 2002); it is frequently associated with overactive bladder syndrome which includes detrusor activity + / - impaired contractility of the bladder. Stress urinary incontinence is the complaint of involuntary leakage of urine on effort or exertion, or on sneezing or coughing (Abrams *et al.*, 2002) and is usually associated with urethral closure incompetence. Mixed urinary incontinence is the complaint of involuntary leakage of urine associated with urgency and also with exertion, effort, sneezing or coughing (Abrams *et al.*, 2002) and is associated with both overactive bladder syndrome and urethral closure incompetence.

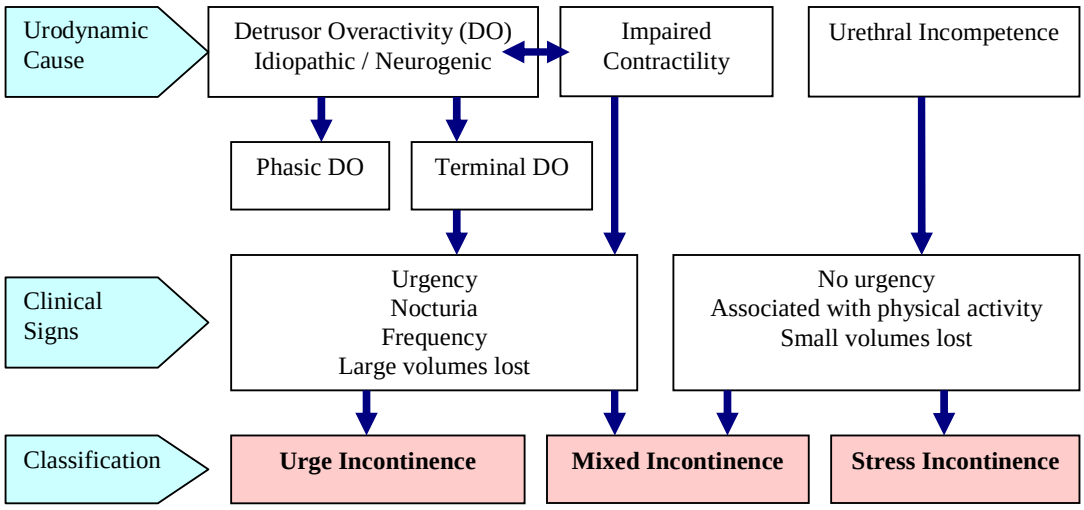


Figure 1-1. Schematic showing the link between the urodynamic causes of urinary incontinence in women, the clinical signs noted by the patient and the classification of the condition.

Detrusor overactivity is a urodynamic observation characterised by involuntary detrusor contractions during the filling phase of micturition (Parsons *et al.*, 2003). Detrusor overactivity can occur with or without a feeling a urgency, approximately a third of patients have an overactive bladder with urgency, termed overactive bladder wet, whilst the rest of patients do not suffer from feelings of urgency and this condition is termed overactive bladder dry (Tubaro, 2004). The amount of urine lost following detrusor overactivity is usually large and symptoms may include urgency, increased frequency of urination and nocturia (Knight-Klimas, 2004). Detrusor overactivity due to neurogenic disease is termed neurogenic detrusor activity or hyperreflexic bladder and any neural lesion or condition that stops the cortical inhibition of detrusor contractions such as spinal cord injury or multiple sclerosis can cause it (Abrams *et al.*, 2002). More commonly a specific cause of detrusor overactivity cannot be found and the condition is then termed idiopathic detrusor activity or detrusor instability (Abrams *et al.*, 2002; Knight-Klimas, 2004; Parsons *et al.*, 2003). Whatever the cause of detrusor overactivity, the involuntary detrusor contractions can be spontaneous or provoked. Spontaneous detrusor overactivity can be sub-defined as phasic or terminal. Phasic detrusor overactivity is characterised by spontaneous contractile activity but does not always lead to clinical urinary incontinence. Phasic detrusor overactivity is defined by a characteristic waveform of contractile pressure mimicking the normal voiding cycle during cystometry. Terminal detrusor overactivity is again spontaneous and is defined as a single involuntary detrusor contraction at

cystometric capacity, which cannot be suppressed and which leads always to clinical signs of urinary incontinence (Abrams *et al.*, 2002; Parsons *et al.*, 2003). Provoked detrusor overactivity occurs in response to either physical or psychological provocation of the bladder such as laughing or hearing running water and may be learnt (Parsons *et al.*, 2003). As toilet training in humans and canines is the learning of cortical inhibition of detrusor contractions, it has been postulated that idiopathic detrusor overactivity is an un-learning or altered learning of this control; therefore altered mental status can allow uninhibited normal detrusor contractions to occur giving the presenting clinical signs of detrusor overactivity without any underlying bladder pathology.

A further condition which can complicate urge incontinence, possibly as a separate entity, or possibly as an end stage of detrusor overactivity is low bladder contractility, often termed impaired detrusor contractility (Elbadawi *et al.*, 1993a; Parsons *et al.*, 2003; Resnick *et al.*, 1987; Zhu *et al.*, 2001). In this condition, the strength of the contractile force of the detrusor is decreased along with a decrease in the sustainability of the contractions (Griffiths *et al.*, 2002). This in turn can lead to an increase in the residual volume of urine left within the bladder after voiding (Griffiths *et al.*, 2002; Resnick *et al.*, 1987), which can cause irritation of the urinary bladder and a propensity to develop secondary bacterial infections, both of which can add to the clinical signs of urinary incontinence.

Stress urinary incontinence is the involuntary leakage of urine through the urethra during periods of raised intra-abdominal pressure, such as coughing or laughing, in the absence of a detrusor contraction and can only be accurately diagnosed after performing urodynamic studies (Abrams *et al.*, 2002; Parsons *et al.*, 2003). Stress urinary incontinence differs symptomatically from overactive bladder syndrome in that the amount of urine lost is often small, nocturia is often absent and there is a lack of urgency (Sarkar *et al.*, 2000). Normally, the urethra remains closed during the filling phase of micturition, maintaining a positive urethral closure pressure even in the presence of raised intra-abdominal pressure, although detrusor overactivity may overcome it (Abrams *et al.*, 2002). An incompetent urethra, however, often considered to be caused by sphincter deficiencies, does not manage to maintain this tight seal, even in the absence of a detrusor contraction and allows the free passage of urine. In women, weak pelvic floor muscles are thought to contribute to the incompetence of the urethra and damage to the levator ani muscles and pubo-urethral ligaments can allow passage of the bladder neck and proximal urethra into the vagina when intra-abdominal pressure increases, thus taking them out of the intra-abdominal pressure zone and further complicating the condition. Factors that may result in such damage

include multiple childbirths, difficult childbirths, obesity, pelvic surgery and chronic cough (Knight-Klimas, 2004; Parsons *et al.*, 2003; Wilson *et al.*, 2003).

Mixed urinary incontinence is a combination of urge and stress incontinences which can act in synergy with each other to cause a more severe and frequent clinical condition. The causes of mixed urinary incontinence are the same as those for the individual syndromes (Abrams *et al.*, 2002; Knight-Klimas, 2004). The condition requires accurate diagnosis, often via cystometry and urodynamic studies and treatment often requires to be tailored to the individual depending on which syndrome predominates.

1.4.2 In the Man

Although urinary incontinence is less prevalent in men than in women the clinical signs and decreased quality of life that patients suffer are comparable. The pathophysiology of urinary incontinence in men differs slightly from that of women, mainly due to the varying anatomy of the urethra and accessory sex glands such as the prostate, however, urodynamically there are a number of similarities between incontinence in men and women. In men urge incontinence due to detrusor overactivity predominates; stress incontinence is rarely seen and then usually in patients who have had prostate surgery or abdominal trauma (Johnson *et al.*, 1999). Reported symptoms of urgency and urge incontinence may be particularly difficult to interpret clinically in men because they might indicate detrusor instability or bladder outlet obstruction causing uninhibited contractions (Hyman *et al.*, 2001; Johnson *et al.*, 1999).

Detrusor overactivity in men has many similarities to that of women with men suffering from both neurogenic and idiopathic detrusor overactivity, the latter being termed detrusor instability. Detrusor instability can be accompanied by low bladder compliance which can lead to residual urine retention, the feeling of incomplete bladder emptying upon urination and increased frequency of urination, as well as the sensation of urgency (Hyman *et al.*, 2001). The feeling of incomplete bladder emptying and increased frequency of urination can also be due to bladder outlet obstruction, however, a condition that has been reported to occur in the presence and absence of detrusor instability (Hyman *et al.*, 2001). In man, the most common cause of bladder outlet obstruction is prostate disease which causes an increase in the size of the prostate which in turn constricts the lumen of the urethra causing a physical obstruction (Ellerkmann *et al.*, 2003). The most common cause of an enlarged

prostate in men is benign prostatic hyperplasia, a condition that occurs with aging, although tumours and infection of the prostate cause the same urodynamic symptoms (Ourad *et al.*, 2003).

In cases of severe prostate enlargement, especially due to neoplasia, the prostate may be surgically resected or removed, which may leaded to development of initial urge incontinence, followed by long term iatrogenic stress incontinence (Fowler *et al.*, 1995; Penson *et al.*, 2008; Rassweiler *et al.*, 2006). Although the type of surgery (radical prostatectomy versus transurethral resection) can influence the likelihood of urinary incontinence developing, with the more extensive surgeries having the highest incidence of post surgical incontinence, the urodynamic findings are the same, with unconscious urine leakage, especially at times of physical effort, being reported by the patient.

1.4.3 In the Bitch

Although there are a number of underlying reasons for urinary incontinence in the canine, as shown above, and whilst multiple causes of urinary incontinence have been characterised in humans, the majority of research into the condition in canines has focused on the role of the urethra. This has led to urethral incompetence, often referred to as urethral sphincter mechanism incompetence, being considered the principle mechanism behind urinary incontinence in the canine (Holt, 1987). In urethral sphincter mechanism incompetence, the resting tone, and therefore resistance produced by the urethra, is decreased and is insufficient to overcome any increases in pressure within the bladder, resulting in urine leakage (Gregory *et al.*, 1996; Rosin *et al.*, 1981). It has been demonstrated, using microtransducers, that urethral closure pressures in incontinent bitches are much lower than in continent bitches and that the urethral closure pressures of bitches decrease significantly after spaying (Reichler *et al.*, 2004; Rosin *et al.*, 1981). The exact reason for this decrease in urethral tone is as yet not known but is hypothesised to be hormonally related as regardless of the procedure used to neuter a bitch her ovaries are removed thus altering her reproductive and sexual hormone production.

Although the urethra appears to play a significant role in the development of urinary incontinence in the neutered bitch, a low urethral resting tone is not a defining characteristic of the condition (Holt, 1998) therefore it is hypothesised that other factors

may be involved. Given the similarities between neutered bitches and post-menopausal women it is feasible that these further complicating factors involve the urinary bladder.

1.4.4 In the Dog

Urinary incontinence in the dog is rarely reported and is believed to be uncommon (Aaron *et al.*, 1996). There is very little published research into the condition but that which is available has focused on the role of the urethra, claiming similarities with urethral sphincter mechanism incompetence in the bitch (Aaron *et al.*, 1996). As in bitches, male canines suffering from urethral incompetence have clinical signs of stress incontinence with urine leakage occurring at times of increased intra-abdominal pressure (Aaron *et al.*, 1996; Power *et al.*, 1998). There is also a reported link between neutering and the development of urinary incontinence in the male canine. The mechanism(s) by which it is thought that neutering leads to acquired urinary incontinence however varies between the sexes (Holt, 1983) as in the male canine castration is associated with a small prostate which may lead to the development of an intra-pelvic bladder and shorter urethra (Power *et al.*, 1998). Both of these anatomical variations lead to a decrease in the urethral closure pressure leading to clinical signs of urinary incontinence (Power *et al.*, 1998). As in men, the prostate can also become enlarged in entire canines, and this can occasionally lead to bladder outlet obstruction and associated urinary incontinence (Holt, 1983).

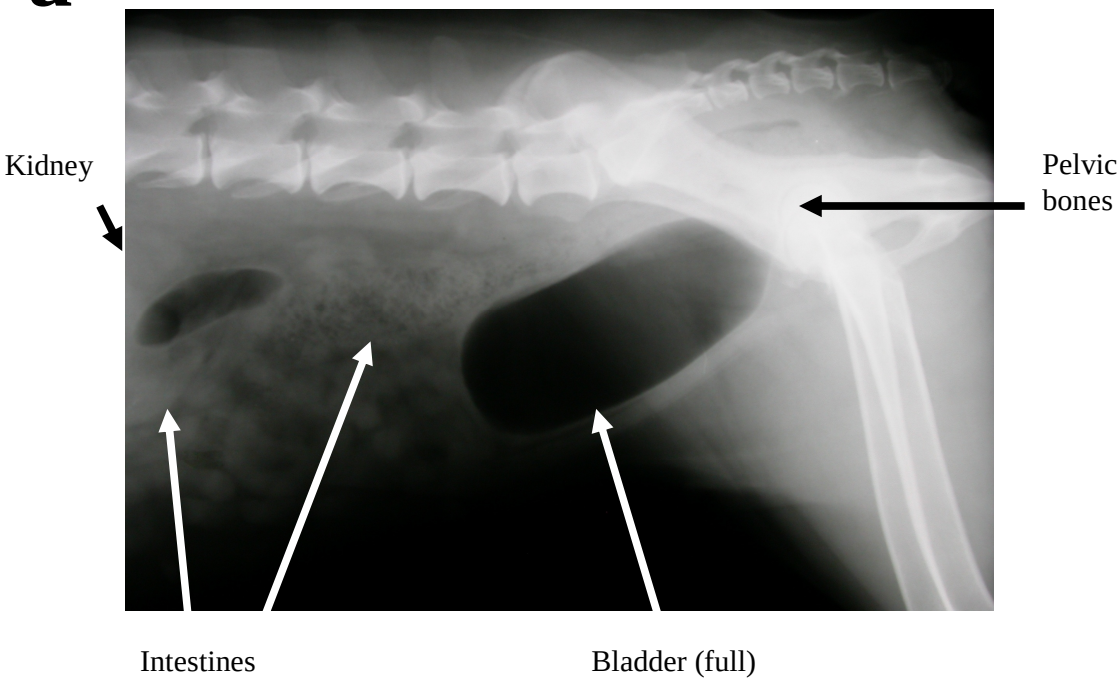
To begin to fully understand and appreciate the mechanisms responsible for the development of urinary incontinence in both bitches and women it is necessary to have an understanding of the anatomy and innervation of the lower urinary tract, and a perception of the main receptors responsible for normal micturition. The sections that follow give an overview of these areas in the bitch, with differences in the woman recorded where appropriate.

1.5 The Anatomy of the Urinary Bladder and Urethra

The urinary system comprises of the paired kidneys that form urine by filtration of the blood; the ureters that convey the urine away from the kidneys; the bladder which is the main storage reservoir for urine; and the urethra along which the urine is discharged. As previously discussed, urinary incontinence can be caused by malfunction of any part of the urinary system; however, only the bladder and urethra have been implicated in the development of acquired urinary incontinence, therefore, this review will focus on these organs.

The urinary bladder is a hollow musculomembranous organ that can change shape, size and position depending on the quantity of urine stored within it (Evans *et al.*, 1993), and the amount of faeces within the rectum (Gray, 2000). The urinary bladder can nominally be split into two sections, an intrapelvic neck region which is continuous with the urethra, and the larger globular body portion that will change relative position within the abdomen depending on the degree of distension with urine. The area of the body of the bladder found most cranially is often referred to as the apex or cranial vertex of the bladder (Jacob, 2007). When empty, the urinary bladder is small and globular, with a very substantial wall (up to 2cm thick) and a negligible lumen (Dyce *et al.*, 2002). The contracted bladder rests on the pubic bones and is entirely intrapelvic in position (Gray, 2000; Smith, 1999). When the bladder relaxes and distends with urine the walls become thinner (down to 2mm thick) and it protrudes cranially, assuming a rough pear shape. As the bladder distends the neck area, which joins to the urethra, remains fixed within the pelvic cavity whilst the body and apex of the bladder extends cranially; in the canine, ventrally, in the human, forwards into the abdomen (Gray, 2000) (Fig. 1-2). The extent of this cranial extension depends on the degree of bladder fill, with an exceptionally full bladder able to reach as far forward as the umbilicus in the canine (Dyce *et al.*, 2002; Evans *et al.*, 1993). When distended, the dorsal (canine) or posterior (human) portion of the bladder is in contact with the jejunum, descending colon and either the cervix and uterine body in the female or the deferent ducts in the male (Evans *et al.*, 1993; Gray, 2000; Jacob, 2007; Smith, 1999). In the canine, the ventral portions of the bladder lie on the visceral and parietal layers of the peritoneum as they overlie the ventral abdominal wall, with the greater omentum occasionally moving caudally to sit between the peritoneal layers (Dyce *et al.*, 2002). In the human, the anterior portions of the bladder rest against the pelvic bones and the anterior abdominal wall (Gray, 2000; Jacob, 2007).

a



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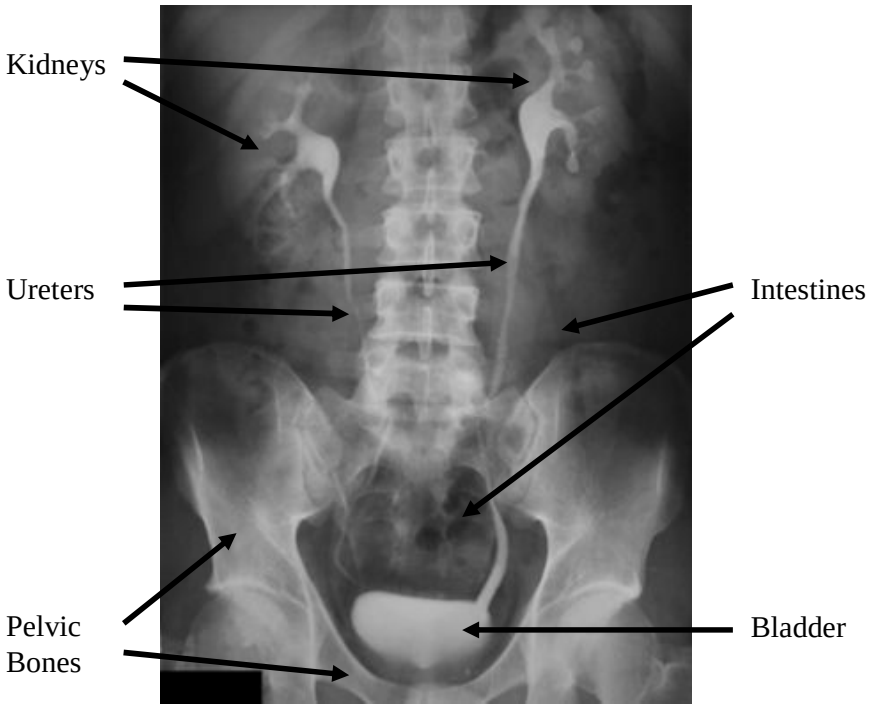

Figure 1-2. Radiograph showing a, lateral view of the abdomen of a bitch (pneumocystogram) and b, an anterior-posterior view of the abdomen of a woman (intravenous pylogram / excretory urogram), demonstrating the normal position of the organs of the urinary system. Photos by kind permission of Glasgow University's Diagnostic Imaging Department.

The urinary bladder is covered with a serosal layer (peritoneum) which extends into three reflections or folds that attach the bladder to the abdominal and pelvic walls and are known as the ligaments of the bladder (Gray, 2000). These are made up of double layers of peritoneum between which are blood vessels, nerves, lymphatics, adipose tissue, ureters, ductus deferens and vestiges of embryonic structures (Evans *et al.*, 1993). The largest fold is the median ligament of the bladder, or median vesicular fold, which runs ventrally from the bladder to the symphysis pelvis and the mid-ventral line of the abdominal wall as far cranially as the umbilicus. The ligament is triangular in shape being widest caudally and narrowing as it moves cranially (Gray, 2000). In the foetus, the median ligament contains the urachus, which normally disappears shortly after birth leaving only the peritoneal fold. The paired lateral ligaments of the bladder or lateral vesical folds, run dorsolaterally from the lateral bladder wall to attach to the abdominal and pelvic walls (Dyce *et al.*, 2002; Evans *et al.*, 1993; Jacob, 2007). These ligaments contain the round ligaments of the bladder in the adult (umbilical artery in the foetus) and the ureters. *In utero* the umbilical arteries, branches of the internal iliac arteries, carry blood from the foetus to the placenta and form part of the umbilical cord. When the cord is severed at birth the arteries retract and contract to become fibrous cords between the bladder and the umbilicus. In the adult, the narrow patent lumen of each vessel still carries blood from the internal iliac artery to the cranial portions of the bladder (Dyce *et al.*, 2002). In the human, the bladder is also connected to the pelvic wall by the fascia endopelvina. In front, this fascial attachment is strengthened by a few muscular fibres, the pubovesicales, which run from the pubic bones to the front of the bladder; at the back, more muscular fibres run from the neck of the bladder to the sides of the rectum and constitute the rectovesicales (Gray, 2000).

The bladder body can be further divided into the domed apex and the body proper (Fig. 1-3). The ureters enter obliquely into the caudal bladder body on its dorsal surface. If an imaginary line was to be drawn between these openings and then from each opening towards the urethra a triangle would be formed. This triangle is known as the trigone area and is thought to have a different embryonic origin from the remainder of the bladder wall (Dyce *et al.*, 2002). The mucosa of the bladder is only loosely attached to the underlying muscularis and has an enormous capacity to stretch with distension of the bladder. The mucosa lining the trigone is thrown into ill-defined ridges that are directed towards the urethra and which form the median urethral crest that continues into the pelvic urethra for a short distance. These ridges are not abolished by distension, unlike the numerous mucosal folds that cover the rest of the bladder's internal surface (Gray, 2000; Smith, 1999).

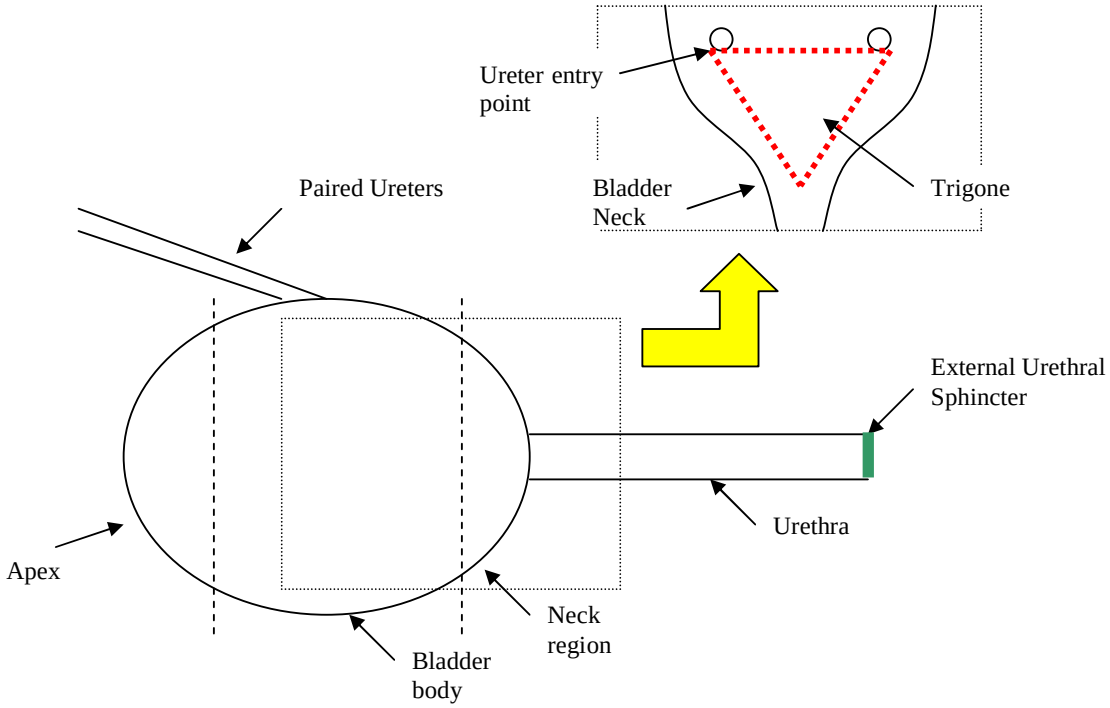

Figure 1-3. Schematic diagram of the bladder and urethra. The trigone area is marked on a cut away diagram viewed from the inside of the bladder in the area shown.

The bladder is composed of four coats or sections, the tunica serosal (outermost), tunica muscularis, tela submucosa and the tunica mucosa which is the inner most layer (Gray, 2000; Henrikson, 1993). The outer tunica serosal is derived from the peritoneum and is the structure reflected onto the abdominal and pelvic walls as discussed above. The tunica muscularis consists of three smooth muscle layers, collectively called the detrusor muscle, with some collagen fibres interspersed. Gap junctions between bladder smooth muscle cells allow for transmission of nerve impulses from cell to cell (Andersson *et al.*, 2004a; Christ *et al.*, 2003). Originally the layers of muscle bundles within the bladder wall were thought to merge at the bladder neck and form an internal urethral sphincter (Wrobel *et al.*, 1993), however, it is now thought that some of the muscle bundles from the bladder wall in the human run directly towards the bladder neck, therefore expanding the urethral opening when they contract (Gray, 2000). This is consistent with the finding in the canine that the proximal urethra may form part of the urine reservoir, expanding when the bladder is distended (Dyce *et al.*, 2002). This means that continence and urethral closure depends on the tension passively exerted by the elastic component of the urethral mucosa, and on the external urethral sphincter formed by the striated urethralis muscle. The tela submucosa consists of a layer of areolar tissue with collagen and elastic fibres, as well as

myofibroblasts (Fry *et al.*, 1997), within it. This layer connects the muscular and mucous coats and is intimately linked to the latter (Gray, 2000; Henrikson, 1993). The inner most coat is the tunica mucosa, a thin lining of the bladder formed by transitional epithelium called urothelium (Henrickson, 1993). It is continuous with the ureters and lining membrane of the renal tubules, as well as with the urethra (Gray, 2000).

The detrusor muscle is the component of the urinary bladder responsible for the mechanical actions of contraction and relaxation. The detrusor muscle is made up of smooth muscle cells arranged into 3 distinct layers which are arranged in an outer and inner longitudinal layer with a middle circular layer (Dyce *et al.*, 2002; Gray, 2000). These muscle bundles may interweave with each other (Henrickson, 1993). In the human detrusor, bundles of muscle cells of varying diameter are surrounded by connective tissue rich in collagen. The bundles of muscle cells in the human detrusor are large and are often composed of several smaller sub-bundles (Andersson *et al.*, 2004a). Within the main bundles of both species the muscle fibres are grouped into small functional units or fascicles (Dyce *et al.*, 2002). The orientation and interaction between the smooth muscle cells in these bundles are important as this will determine some of the properties of the bladder wall and its behaviour, such as the shape of the bladder and its intraluminal pressure (Andersson *et al.*, 2004a). In smaller animals, such as the rat, the muscle bundles are less complex and the patterns of arrangement simpler than in the human detrusor (Gabella *et al.*, 1990). There have been no comparison studies in the canine looking at the fine structure of the urinary bladder; however it is hypothesised to be similar to that of the human. Figure 1-4 shows the gross structure of the canine urinary bladder wall, demonstrating the arrangement of the muscle layers and bundles which appears to be similar to that described for the human.

The individual smooth muscle cells that make up the detrusor muscle in humans and canines are typical of urinary smooth muscle cells throughout the mammalian body. They are long, spindle shaped cells with a central nucleus. The cytoplasm is packed with myofilaments, and the membranes contain regularly spaced dense bands with membrane vesicles between them. Mitochondria and a few elements of sarcoplasmic reticulum are also present, mainly near the nucleus (Andersson *et al.*, 2004a; Henrikson, 1993).

During filling of the bladder, in the storage phase of micturition, the smooth muscle cells have to relax and to elongate and rearrange in the wall of the bladder over a significant period of time (hours). During the emptying phase of micturition, the smooth muscle cells of the bladder must shorten quickly (seconds) and generate large forces in a synchronous

manner to allow fast and smooth bladder contraction. These activities of the detrusor muscle require regulation of both contraction and relaxation via nervous and hormonal control systems (see sections 1.6-1.11).

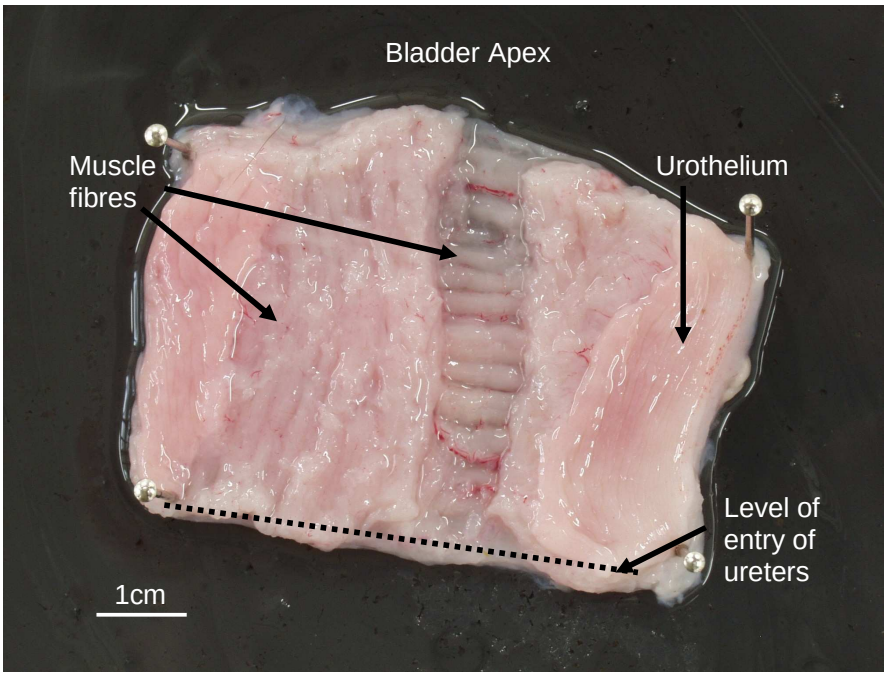

Figure 1-4. Photograph showing the different layers and orientations of the muscle fibres that make up the detrusor muscle of the canine bladder. Photo courtesy of Richard Irving, Division of Pathological Sciences, Glasgow Vet School.

Early work on smooth muscle organs divided smooth muscle into two distinct classes termed single-unit and multiunit on the basis of contractile behaviour (Bozer, 1941). Single-unit smooth muscles are arranged in bundles with many points of close contact between the cell membranes, gap junctions. Gap junctions are low resistance pathways through which ions can pass from one cell to another, thereby rapidly spreading an electrical signal throughout a tissue. A small number of fibres in a single-unit muscle will spontaneously depolarise generating an action potential, these are called pacemaker cells (Andersson *et al.*, 2004a). In single-unit smooth muscles these pacemaker cells can often be stimulated to produce an action potential when the muscle is stretched. Multiunit smooth muscles are composed of discrete muscle bundles and fibres that operate independently from each other. They tend to be well innervated by the autonomic nervous system and are controlled mainly by direct nerve signals, therefore, they rarely show spontaneous contractions (Bozer, 1941). The detrusor muscle exhibits characteristics of

both single-unit and multiunit muscles, with electrical coupling between cells and spontaneous action potentials developing, yet being densely innervated and requiring nervous coordination to achieve voiding (Andersson *et al.*, 2004a).

In the female the urethra runs caudally from the bladder neck to the external body surface along the pelvic floor. It passes through the vaginal wall at an oblique angle and opens ventrally at the vaginal-vestibule junction (Gray, 2000). In the male the urethra runs from the bladder neck to an external orifice at the free extremity of the penis and can be split into two sections: an internal pelvic portion and an external or spongy part, the latter named for the very vascular tissue that surrounds the urethra on its leaving the pelvic cavity. The first part of the urethra in the male is surrounded entirely by the prostate and is penetrated by the opening of the deferent ducts and the numerous pores that drain the prostate (Dyce *et al.*, 2002; Gray, 2000). The urethral lumen widens caudal to the prostate but gradually narrows again as it approaches the ischial arch over which it exits the pelvic region (Dyce *et al.*, 2002).

The urethra has a similar structure to that of the bladder with a transitional epithelial lining, a middle muscular layer and an outer serosal layer. As the urethra progresses towards the external urethral orifice the transitional epithelium gives way to stratified squamous epithelium (Gray, 2000). The muscle bundles are less well arranged than that of the bladder but contain both longitudinal and circular components (Henrickson, 1993). In both the female and the male, the smooth muscle gradually gives way to skeletal (striated) muscle, the urethralis muscle, along the length of the urethra, the change happening more distally in the female than in the male (Smith, 1999; Wrobel *et al.*, 1993). The external urethral sphincter is formed of this skeletal muscle and as such is near the junction of the urethra and the vestibule in female, and is found along the length of the urethra from just caudal of the prostate to the root of the penis in males (Smith, 1999). Mixed in with these fibres are muscle fibers from the rhabdosphincter, a striated muscle that sits in a horseshoe shape around and under the urethra and that helps to close the urethral lumen when it is contracted (Strasser *et al.*, 2000). The urethral submucosa contains a complex venous network that is thought to constitute a form of erectile tissue in the female as well as the male; this may help improve continence by assisting mucosal apposition and abolishing the lumen of the urethra during the storage phase of micturition (Dyce *et al.*, 2002; Gray, 2000).

The pelvic floor or pelvic diaphragm is found in all animals and although is not a true part of the urinary system is often implicated in the development of stress incontinence in

women (Rogers, 2008). The pelvic diaphragm is composed of muscle fibers of the levator ani muscles, the coccygeus muscle and associated connective tissue which span the area posterior the pelvis; it separates the pelvic cavity cranially from the perineal region caudally (Jacob, 2007). The right and left levator ani lie across the floor of the pelvis and are separated by a narrow gap that transmits the urethra, vagina and anal canal. The levator ani is usually considered in three parts: pubococcygeus, puborectalis, and iliococcygeus (Dyce *et al.*, 2002). The pubococcygeus is the main part of the levator ani and runs from the body of the pubis to the coccyx, incorporating some fibers into the prostate, urethra and vagina (Gray, 2000). The right and left puborectalis unite behind the anorectal junction to form a muscular sling which some experts regard as part of the external anal sphincter (Gray, 2000). The iliococcygeus, the most posterior part of the levator ani, is often poorly developed. The coccygeus, situated behind the levator ani is frequently composed of tendinous as well as muscular fibers; it extends from the ischial spine to the lateral margin of the sacrum and coccyx (Gray, 2000). Damage to any part of the pelvic diaphragm, for example during parturition or pelvic surgery, can result in both urinary and faecal incontinence, as well as prolapse of the abdominal organs.

The blood supply to the bladder is through the cranial and caudal vesical arteries (Evans *et al.*, 1993; Gray, 2000; Smith, 1999). The cranial vesical artery is a branch of the umbilical artery and supplies the cranial portion of the bladder including the apex and part of the dome (Dyce *et al.*, 2002; Evans *et al.*, 1993). The caudal vesical artery is a branch of the vaginal or prostatic artery from the internal iliac artery and supplies the caudal part of the dome and the neck of the bladder (Dyce *et al.*, 2002; Smith, 1999). There is a venous plexus on the surface of the bladder that drains primarily into the internal pudendal veins (Evans *et al.*, 1993; Gray, 2000; Smith, 1999).

The blood supply to the urethra varies slightly with the sexes as the urethra travels caudally. The cranial portion in both sexes, which extends to the whole of the urethra in females, is supplied by the paired urethral arteries that are branches of the vaginal or prostatic artery, themselves branches of the internal iliac artery (Dyce *et al.*, 2002; Evans *et al.*, 1993; Gray, 2000). A branch of the artery of the penis supplies the penile portion of the male urethra; it is a terminal branch of the internal pudendal artery (Dyce *et al.*, 2002). The venous drainage is via the satellite veins of the aforementioned arteries (Dyce *et al.*, 2002; Gray, 2000; Smith, 1999).

1.6 Innervation of the Lower Urinary Tract

Micturition and urine storage depend on coordinated action between two functional units in the lower urinary tract; a reservoir (the urinary bladder) and an outlet (the bladder neck and the smooth and striated muscle of the urethra) (de Groat *et al.*, 2001). During voiding, the muscles at the bladder outlet relax and the bladder smooth muscle contracts, raising the intravesical pressure and inducing urine flow. During urine storage, the bladder outlet is closed and the bladder smooth muscle is quiescent, allowing the intravesical pressure to remain low over a wide range of bladder volumes. These changes are coordinated by three sets of nerves (parasympathetic, sympathetic, and somatic) that emerge from the sacral and thoracolumbar spinal cord (Andersson, 1993) (Fig. 1-5).

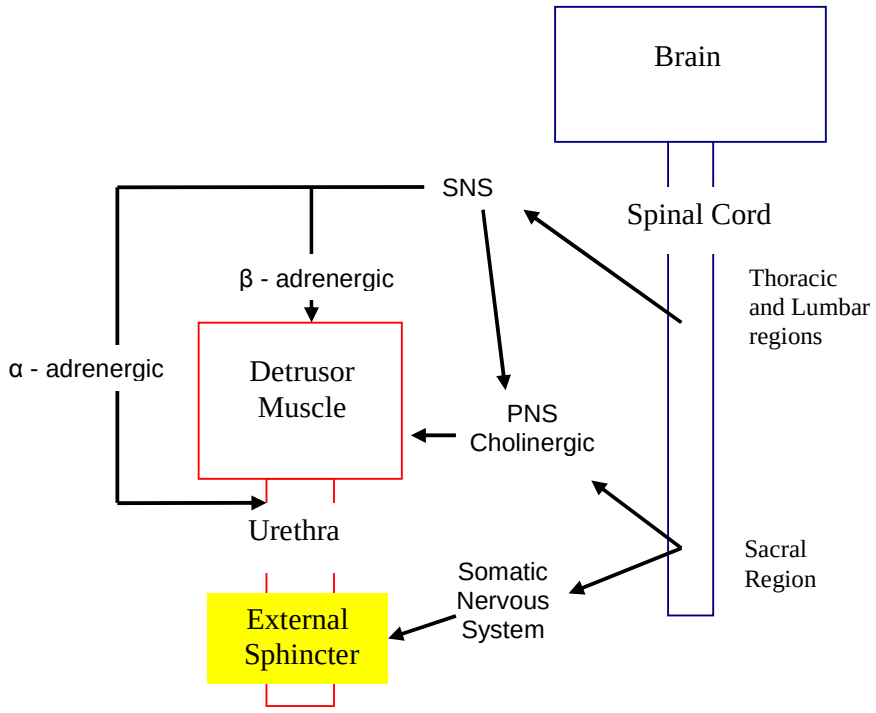

Figure 1-5. Schematic diagram showing the innervation of the lower urinary tract. SNS is the Sympathetic Nervous System, whilst PNS is the Parasympathetic Nervous System.

The hypogastric nerve, originating from spinal cord segments of L1 through L4, supplies sympathetic innervation to the bladder and urethra, as well as the rhabdosphincter (Creed, 1979; Cunningham, 1992; Evans *et al.*, 1993). The pelvic nerve, originating from the spinal cord segments S2 through S4, supplies parasympathetic (cholinergic) innervation to the detrusor muscle and transmits sensory impulses from the bladder (Cunningham, 1992; Evans *et al.*, 1993; Smith, 1999). Somatic innervation of the muscle of the external urethral

sphincter is distributed via the pudendal nerve, originating from spinal cord segments S1 through S3 (Evans *et al.*, 1993; Smith, 1999). The pudendal nerve also innervates muscles of the anal sphincter and perineal region (Dyce *et al.*, 2002). The spinal cord carries messages to and from the brain to accomplish voluntary control over micturition. The micturition reflex centre has been localized in the pontine-mesencephalic reticular formation in the brainstem (de Groat, 1998). There are interconnections from the micturition reflex centre to the frontal lobes and other areas in the cortex and subcortical areas (de Groat, 1990).

The sympathetic and somatic nervous systems dominate during the storage phase of micturition (Evans *et al.*, 1993; Longhurst *et al.*, 2001; Smith, 1999). Sympathetic stimulation via β-adrenoceptors in the detrusor muscle results in bladder relaxation to accommodate filling. Sympathetic stimulation via α-adrenoceptors in the neck of the bladder results in contraction and closure of the sphincter that maintains continence (de Groat, 1998). Sympathetic pathways, acting via tonic inhibitory systems in the micturition centre of the rostal pons, also inhibit parasympathetic bladder innervation during storage (de Groat, 1998). Stimulation of the pudendal nerve results in increased tone of the external urethral sphincter, contributing to continence (Creed, 1995). External urethral sphincter tone can also increase in response to sudden increases in abdominal pressure (during coughing or barking) to maintain continence (Brading, 1999; Kamo *et al.*, 2003; Thuroff *et al.*, 1982).

When the bladder is filled with urine, sensation from tension receptors, volume receptors and nociceptors in the bladder wall is transmitted via afferent nerve fibres in the pelvic nerve to the sacral spinal cord and subsequently the brainstem (Andersson, 2002). Voluntary control of urination originates from the cerebral cortex (Blok, 2002; de Groat, 1990; Sugaya *et al.*, 2005). During the emptying phase of micturition, parasympathetic (cholinergic) stimulation of the detrusor muscle results in bladder contraction via muscarinic receptors (Andersson *et al.*, 2004a; Evans *et al.*, 1993; Smith, 1999). Simultaneous inhibition of sympathetic nerves and somatic stimulation of the urethral smooth and skeletal muscle, along with stimulation of muscarinic receptors in the urethra causing nitric oxide release, results in urethral relaxation (Chess-Williams, 2002; Evans *et al.*, 1993; Smith, 1999; Van der Werf *et al.*, 2002). Following complete emptying of the bladder or voluntary cessation of urination, the storage phase begins again.

1.7 Cellular Basis of Contraction

Contraction of the smooth muscle of the detrusor is initiated by an increase in the intracellular Ca^{2+} concentration. It is understood that Ca^{2+} can either enter the cytoplasm through the cell membrane, via Ca^{2+} channels, or be released from the sarcoplasmic reticulum (Andersson *et al.*, 2004a). These pathways for Ca^{2+} translocation can be seen in Figure 1-6.

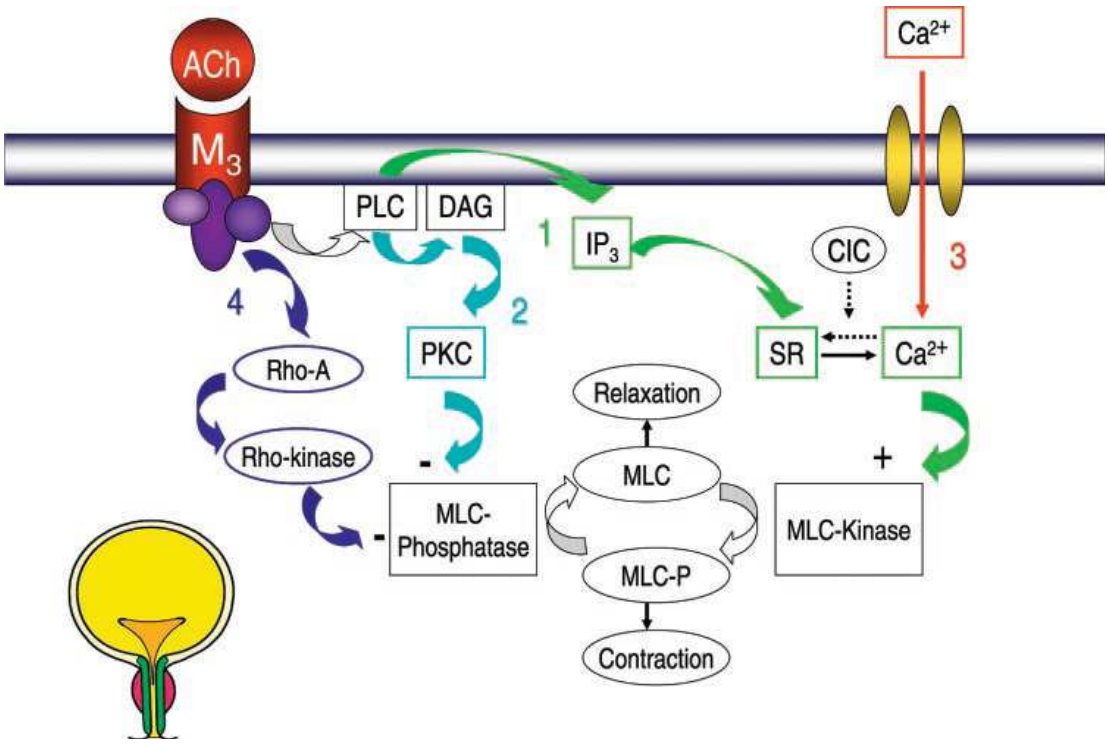

Figure 1-6. Signal pathways (1–4) involved in activation of detrusor contraction via muscarinic M_3 receptors. ACh, acetylcholine; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MLC, myosin light chain; IP_3, inositol trisphosphate; SR, sarcoplasmic reticulum; CIC, calcium-induced calcium release. There seem to be differences between species in the contribution of the different pathways in contractile activation. In human detrusor, Ca^{2+} influx (3) is of major importance. Taken from Urinary Bladder Contraction and Relaxation: Physiology and Pathophysiology: Physiol Rev, Jul 2004; page 958. Copyright The American Physiological Society, used with permission.

The release of Ca^{2+} from the sarcoplasmic reticulum is an important step in activation of the detrusor muscle. The release of Ca^{2+} is triggered by inositol trisphosphate (IP_3) via IP_3 receptors. IP_3 is one of the breakdown products of phosphatidylinositol, a phosphorylated membrane phospholipids; diacylglycerol (DAG) is the other breakdown product. The breakdown is initiated by activation of the muscarinic receptor on the cell wall and the

ensuing G-protein-coupled mechanism which in turn activates phospholipase C. IP_3 can diffuse and bind to specific receptors on the sarcoplasmic reticulum and release Ca^{2+} stored within the organelle into the sarcoplasm (Andersson *et al.*, 2004a).

The Ca^{2+} activation of the contractile proteins is known to occur via a phosphorylation pathway where Ca^{2+} binds to a soluble protein calmodulin, and the Ca^{2+}/calmodulin complex activates the myosin light-chain kinase (MLCK) which catalyzes the phosphorylation of the myosin regulatory light chains (Fry *et al.*, 2002). Dephosphorylation of the regulatory light chain is performed by a myosin light-chain phosphatase (MLCP).

Relaxation of the smooth muscle cells occurs when the intracellular Ca^{2+} concentration is reduced back to the resting level by reuptake via an adenosine triphosphate (ATP)-consuming Ca^{2+} pump (SERCA), into the sarcoplasmic reticulum (Fry *et al.*, 2002).

Despite these mechanisms it is known that some Ca^{2+} is lost across the cell membrane, possible via a Ca^{2+} pump, powered by ATP hydrolysis and an exchange of Ca^{2+} for Na^{+} by a Na^{+}-Ca^{2+} counter exchanger (Wu *et al.*, 2001). This lost Ca^{2+} must be replenished by subsequent Ca^{2+} entry into the cell to ensure a steady state with respect to cellular Ca^{2+}. The replenishment of this intracellular Ca^{2+} is thought to occur by a number of mechanisms including influx via voltage-activated Ca^{2+} channels and Na^{+}-Ca^{2+} exchange (Fry *et al.*, 2002). This influx of Ca^{2+} is exceptionally important as without it the calcium stores of the cell will diminish and contraction of the muscle will no longer be possible.

Although the main mechanisms of contraction of the smooth muscle cells of the detrusor involve Ca^{2+} release from the sarcoplasmic reticulum and influx of calcium via Ca^{2+} channels and Na^{+}-Ca^{2+} exchange it is understood that inhibition of the MLCP can lead to Ca^{2+} sensitization and improved contraction of the cell (Andersson *et al.*, 2004a).

One main pathway for inhibition of the MLCP involves a specific kinase (*Rho*-associated kinase), which is activated via small G proteins of the *Rho* superfamily, in the case of the detrusor *RhoA* (Symons M, 1996). Activation of *Rho*-associated kinase leads to phosphorylation of the myosin binding subunit of the MLCP and inhibits this enzyme. A further pathway for modulating the Ca^{2+} sensitivity of contraction is via the protein kinase C (PKC) (Jensen *et al.*, 1996). This is thought to act via CPI-17 which is strongly phosphorylated by PKC and which in turn inhibits the action of MLCP (Eto *et al.*, 1999).

In conclusion, although there are many pathways that can cause contraction of the smooth muscle cells of the detrusor, the concentration of calcium within the cell is the determining factor in cellular contraction. The release of calcium from the sarcoplasmic reticulum, and the influx of calcium into the cell, is the main factor involved in determining cellular contraction, and the primary receptor responsible for initiating calcium release is the muscarinic receptor, although other receptors such as purinergic receptors also have a role to play.

1.8 Muscarinic Receptors of the Urinary Bladder

As has been previously discussed, the parasympathetic nervous system, acting via the muscarinic receptors, is the main motor drive to the urinary bladder during micturition (Chess-Williams, 2002). Defects in this system, involving the muscarinic receptors, are hypothesised to be involved in the development of urinary incontinence in women (Chess-Williams, 2002), and thus potentially in canines. Muscarinic receptors are activated via acetylcholine (ACh), the neurotransmitter released by the parasympathetic neurones. They are found throughout the bladder but predominate within the smooth muscle layer (Andersson *et al.*, 2004b).

There are five muscarinic receptor subtypes, encoded by five distinct genes (Caulfield, 1993). The five gene products correspond to pharmacologically defined receptors and M_1 – M_5 are used to describe both the molecular and pharmacological subtypes (Caulfield *et al.*, 1998). Muscarinic receptors are all coupled to G proteins, although the signal transduction systems vary. M_1, M_3 and M_5 receptors are coupled preferentially to $G_{q/11}$ proteins which activate phospholipase C leading to mobilisation of intracellular calcium. M_2 and M_4 receptors are coupled to pertussis toxin sensitive $G_{i/0}$ proteins which inhibit adenylyl cyclase (Andersson *et al.*, 2004b). Muscarinic receptors are found throughout the body, however, not all receptors have been demonstrated in all tissues (Table 1-2), and the functional role of the receptors in certain tissues has not been fully elucidated. Data from the rat would suggest that receptor abundance is directly associated with contractile ability as it is known that in the rat the density of muscarinic receptors is greatest in the dome of the bladder and lowest in the neck region of the bladder (Saito *et al.*, 1997) and the greatest contraction following muscarinic receptor stimulation occurs in the dome (Levin *et al.*, 1988). Within the wall of the human urinary bladder, muscarinic receptors can be found on the detrusor muscle itself (Chess-Williams, 2002), where they cause contraction of the bladder, on the urothelium (Chess-Williams, 2002) where they are thought to cause the release of a diffusible factor that may inhibit contraction and on autonomic nerve endings where they alter transmitter release (Chess-Williams, 2002). There have been no reported studies looking at the distribution of muscarinic receptors in the canine bladder; although given the distribution in other species such as the rat (Saito *et al.*, 1997) it can be hypothesised that muscarinic receptors in the canine urinary bladder will closely mimic those in their human counterparts.

	G-protein	Cellular response	Main locations
M_1	$G_{q/11}$	↑ IP3, DAG	CNS (cerebral cortex)
		Excitation	Glands (gastric, salivary etc)
M_2	$G_{i/0}$	↓ cAMP	CNS (widely distributed)
		Inhibition	Heart
			Smooth muscle (bladder, gastrointestinal tract)
M_3	$G_{q/11}$	↑ IP3	CNS (widely distributed)
		Stimulation	Glands (gastric, salivary etc)
			Smooth muscle (bladder, airways, gastrointestinal tract, eye)
			Endothelium
M_4	$G_{i/0}$	↓ cAMP	CNS (cortex, hippocampus)
		Inhibition	
M_5	$G_{q/11}$	↑ IP3	CNS (Substantia nigra)
		Excitation	Iris / ciliary muscle

Table 1-2. Table showing the main properties and locations of the muscarinic receptor subtypes in the mammalian body. IP3, inositol triphosphates; DAG, diacylglycerol; cAMP, cyclic adenosine-3',5'-monophosphate; CNS, central nervous system.

The muscarinic receptor subtypes reported within the bladder vary between species and between studies, however all five receptor subtypes have now been demonstrated within the human bladder (Sigala *et al.*, 2002) whilst the M_1-M_4 receptor subtypes have been reported in the rat urinary bladder (Braverman *et al.*, 1998a), and only the M_2 and M_3 receptor subtypes have been demonstrated in the pig (Maeda *et al.*, 1988; Yamanishi *et al.*, 2000) and rabbit (Wang *et al.*, 1995). In most species so far studied, the M_2 receptor subtype outnumber the M_3 receptor subtype in the urinary bladder by 3:1, however, in the rat this ratio is increased to 9:1 (Chess-Williams, 2002).

Despite this disparity in receptor numbers it is the M_3 receptor subtype that is thought to be the main functional receptor responsible for bladder contraction during micturition (Chess-Williams, 2002). This has been demonstrated in pharmacological characterisation studies in normal bladder tissue from humans (Chess-Williams *et al.*, 2001), pigs (Sellers *et al.*, 2000), rats (Braverman *et al.*, 1998a; Braverman *et al.*, 1998b; Longhurst *et al.*, 2000), rabbits (Barras *et al.*, 1999) and monkeys (Lai *et al.*, 1998). However there is thought to be a minor role played by the more numerous M_2 receptors in bladder contraction, as shown by the decreased responsiveness of detrusor muscle strips to muscarinic stimulation *in vitro* in tissue collected from M_2 knock-out mice (Stengel *et al.*, 2000). *In vivo* it has

also been shown via intravesicular pressure measurements in muscarinic antagonist treated anesthetised rats that M_2 receptors are involved in micturition (Hegde *et al.*, 1997), however, their exact role in normal micturition has not yet been determined. In animals with a low M_2:M_3 receptor ratio such as the pig or human it has been postulated that M_2 receptors may regulate smooth muscle tone under certain conditions such as high sympathetic activity (Eglen *et al.*, 1994). Therefore, while sympathetic activity usually causes relaxation of the bladder via β-adrenoceptors to facilitate urine storage (Levin *et al.*, 1988), activation of M_2 receptors may effectively switch off the sympathetic inhibitory mechanisms mediated by the β-adrenoceptors leading to improved emptying of the bladder. Furthermore M_2 receptors may become more important where there is M_3 receptor dysfunction, for example it has been shown in rats that there is an increase in the density of M_2 receptors within the bladder in certain disease states such as diabetes (Tong *et al.*, 1999) and denervation (Braverman *et al.*, 1999a; Braverman *et al.*, 1998b), however, a similar phenomenon has not been reliably demonstrated in the human bladder (Uchiyama *et al.*, 2004).

All of the subtypes of muscarinic receptor have been demonstrated in the urothelium of humans (Bschleipfer *et al.*, 2007), however, none of the subtypes have been found in the rat urothelium (Gunasena *et al.*, 1995; Saito *et al.*, 1997) which suggests that species differences in the distribution of muscarinic receptors and their control of micturition may occur. It has been shown that the urothelium has an afferent innervation (Wakabayashi *et al.*, 1993) and it is suggested that at least part of this is a parasympathetic motor innervation (Wakabayashi *et al.*, 1995). The exact role of this innervation with regard to micturition has yet to be elucidated but the ACh released has been hypothesised to play a role in the release of the urothelium-derived inhibitory factor described by Hawthorn *et al.* (2000) which is thought to aid in relaxation of the bladder. It has also recently been demonstrated that there is a non-neuronal cholinergic system within the human bladder urothelium that produces ACh (Yoshida *et al.*, 2006). A role for this non-neural ACh has not been demonstrated although a number of hypothesis have been put forward, including an action on the muscarinic receptors within the detrusor muscle to enhance contraction. It is considered more likely, however, that this non-neuronally secreted ACh may stimulate the urothelial muscarinic receptors and affect the sensory pathway during the storage phase of micturition (Yoshida *et al.*, 2006), potentially via the urothelium-derived diffusible inhibitory factor that has been shown to inhibit contraction of the underlying detrusor muscle to a variety of contractile agents in both pigs (Hawthorn *et al.*, 2000) and humans (Chaiyaprasithi *et al.*, 2003).

Finally, muscarinic receptors have also been demonstrated presynaptically on both the parasympathetic and sympathetic nerve endings within the bladder (Chess-Williams, 2002). Stimulation of the prejunctional muscarinic receptors on the parasympathetic nerves have been shown to have both inhibitory and facilitatory effects in a number of species including rats and rabbits (Braverman *et al.*, 1998a; D'Agostino *et al.*, 1997; Inadome *et al.*, 1998; Somogyi *et al.*, 1994). The facilitatory effect is mediated via prejunctional M_1 receptors which increase ACh release (Somogyi *et al.*, 1999), whilst the inhibitory mechanism decreases ACh release and is mediated via the M_2 or M_4 receptors (Braverman *et al.*, 1998a; D'Agostino *et al.*, 1997). Whether the inhibitory or facilitatory mechanism predominates at any given time will depend on a number of factors including stimulation parameters (Somogyi *et al.*, 1999), and may therefore have a role to play in the development of urinary incontinence.

1.9 Adrenoceptors in the Urinary Bladder

The body of the bladder receives only a sparse innervation by noradrenergic nerves from the sympathetic nervous system. The density of these neural inputs increases towards the bladder neck and urethra, especially in the male (Gosling *et al.*, 1999). The functional significance of these nerves, which also occur in the lamina propria of the bladder have not yet been fully established.

Both α-adrenoceptors and β-adrenoceptors have been reported within the urinary bladder of humans (Andersson *et al.*, 2004a); however, the functional importance of the α-adrenoceptors is undetermined. Stimulation of the α-adrenoceptors causes contraction of the detrusor, whilst stimulation of the β-adrenoceptors causes relaxation (Andersson, 1993). It is possible to provoke contraction of the detrusor with sufficiently high concentrations of drugs that act exclusively on the α-adrenoceptors in a number of species, however, the number of α-adrenoceptors in the human bladder is low and the β-adrenoceptors predominate (Goepel *et al.*, 1997). This means that the normal response of the human isolated detrusor muscle to noradrenalin is relaxation (Andersson, 1993). This may mean that the α-adrenoceptors have no significant role in normal bladder contraction in the human, however, it is hypothesised that this may change in certain disease states such as bladder outlet obstruction and overactive bladders, although the results of studies looking at this have not been conclusive (Andersson *et al.*, 2004a).

Noradrenalin is released by electrical stimulation of the adrenergic nerves in detrusor tissue (Mattiasson *et al.*, 1987) and since β-adrenoceptors predominate over α-adrenoceptors post-junctionally in the human bladder relaxation ensues (Andersson, 1993; Nomiya *et al.*, 2003). There are three subtypes of β-adrenoceptor; β_1-, β_2- and β_3-adrenoceptors. All three of these have been demonstrated in the urinary bladder of the human, as well as a number of other species including the monkey (Takeda *et al.*, 2002), ferret (Takeda *et al.*, 2000a), canine and rat (Takeda *et al.*, 2003). The functional subtype, however, appears to vary with species with the guinea-pig having relaxation mediated via the β_1-adrenoceptor (Li *et al.*, 1992), the rat via β_1-, β_2- and β_3-adrenoceptors (Longhurst *et al.*, 1999; Takeda *et al.*, 2000b) and the human by β_3-adrenoceptors.

These findings suggest that β-adrenoceptor stimulation may help keep the bladder relaxed during filling. There have, however, been no reported functional studies *in vivo* to test this hypothesis and the importance of sympathetic input for human bladder control is controversial (Andersson *et al.*, 2004b). It is known that sypathectomy has no significant

effect on bladder filling and neither does blockade of the β-adrenoceptors, therefore the sympathetic nervous system may not be essential for urine storage in humans, although it may play a role in various disease states (Andersson, 1986). In addition a study in rats has demonstrated that a selective β_3-adrenoceptor agonist can increase the voiding interval and decrease the number of spontaneous contractions during the filling phase of micturition in certain models of induced bladder overactivity, leading to the hypothesis that activation of the β_3-adrenoceptor can directly inhibit smooth muscle contractility in this species, with this condition (Woods *et al.*, 2001). Whether or not this will hold true for other species, and whether or not activation of β_3-adrenoceptors is an effective way of treating detrusor overactivity has not yet been reported.

1.10 Non-Adrenergic, Non-Cholinergic Mechanisms of Bladder Control

It is known that in most mammalian species part of the neuronally induced bladder contraction is resistant to atropine (Andersson, 1993), a competitive antagonist of the muscarinic receptors. This atropine resistant component of contraction is termed the non-adrenergic, non-cholinergic mediated response, and its proportion to the total contraction varies with both species and the frequency of stimulation used in *in vitro* studies. It has been reported that atropine can block up to 25% of the contractile response in isolated strips of detrusor muscle from rats (Andersson *et al.*, 2004a), but in rabbits and pigs this figure rises to 40 and 75% respectively (Brading *et al.*, 1991). There have been no reported studies looking at the role of non-adrenergic, non-cholinergic mechanisms in bladder contraction in canines.

In the human bladder the role of the non-adrenergic, non-cholinergic mechanism is still disputed (Andersson *et al.*, 2004a). There have been a number of studies looking at the role of acetylcholine in the electrically induced contraction of isolated strips of detrusor muscle that have variously concluded that the non-adrenergic, non-cholinergic mechanism is responsible for between 0 and 50% of the nerve mediated contraction of the urinary bladder in the human (Cowan *et al.*, 1983; Sibley, 1984). This dramatic difference is likely due to differences in the experimental protocols used, as a further study demonstrated that with an experimental protocol involving only the minimal electrical field stimulation required to produce consistent and reproducible tissue responses, atropine blocked only 70% of the contractile response (Luheshi *et al.*, 1990). With a stimulation protocol involving long trains of large pulses, however, the same study demonstrated 100% atropine sensitivity, demonstrating the need for minimal electrical field stimulation in this type of research and for accurate comparisons between studies to be made, acknowledging what the stimulation parameters for each study are. Further studies looking at human bladder contractility have consistently demonstrated an atropine-resistant, tetrodotoxin-sensitive component to nerve-mediated contraction (Bayliss *et al.*, 1999; O'Reilly *et al.*, 2002; Palea *et al.*, 1993). The contribution of non-adrenergic, non-cholinergic mechanisms to contraction of the normal human bladder is, however, thought it be small.

There have been a number of reported studies looking at the role of the non-adrenergic, non-cholinergic mechanism in bladder contraction in certain disease states in humans, namely bladder outflow obstruction and detrusor overactivity. These have shown varying

results but agree that in bladder outlet obstruction and detrusor overactivity there is an atropine-resistant component of between 25 and 65% of electrically induced contraction (Andersson *et al.*, 2004b), much greater than the 0 to 25% demonstrated in their control tissues. Part of this atropine-resistant component may be due to direct muscle stimulation of hypertrophied detrusor muscle (Tagliani *et al.*, 1997), however, at least some of the atropine-resistant responses were tetrodotoxin sensitive, suggesting a nerve-mediated response (Andersson *et al.*, 2004b).

The functional neurotransmitter(s) responsible for the non-adrenergic, non-cholinergic mechanism have not yet been identified, however, a number of potential compounds have been identified. These include adenosine-5'-triphosphate (ATP), nitric oxide, various neuropeptides and prostanoids. Of these, ATP has been the most extensively studied and demonstrates the most compelling evidence for involvement in the non-adrenergic, non-cholinergic mechanism of bladder contraction (Andersson *et al.*, 2004a).

It has been shown by a number of studies that the atropine-resistant contractile component of nerve-mediated detrusor contraction in humans and guinea-pigs can be blocked by α, β-methylene ATP, an agent known to stimulate and then to desensitize P2-purinoceptors, which suggests that ATP is the non-adrenergic, non-cholinergic mediator in these species (Andersson *et al.*, 2004a; O'Reilly *et al.*, 2002; Palea *et al.*, 1993). It is known that ATP works via stimulation of P2X receptors (Andersson *et al.*, 2004a), and it has been shown that the P2X receptor subtypes are present in the human bladder (Hardy *et al.*, 2000; O'Reilly *et al.*, 2002). Further studies have shown that other animals, such as the rat, mouse, rabbit and cat have multiple purinergic excitatory receptors present within the bladder as well (Andersson, 1993). It is hypothesised that ATP mediates detrusor contraction via activation of a ligand-gated cation channel (the P2X receptor) that promotes the influx of extracellular Ca^{2+} (Andersson *et al.*, 2004a). Changes in the P2X receptor subtypes including selective loss of the $P2X_3$ and $P2X_5$ receptor subtypes have been reported in idiopathic detrusor overactivity (Moore *et al.*, 2001), and the amount of $P2X_1$ receptor, the predominant purinoceptor subtype in the human bladder, has been shown to be greater in patients with obstructed bladders compared to normal bladders (O'Reilly *et al.*, 2002). This all suggests that ATP may contribute to excitatory neurotransmission within the bladder, and that the purinergic system, as part of the non-adrenergic, non-cholinergic mechanism may have a greater role to play in certain disease states.

1.11 Reproductive Hormones in the Woman

In the woman, cyclic ovarian function, often referred to as menstrual cycles, occur between puberty and menopause, which are increasing and decreasing periods of ovarian activity respectively. The average length of each menstrual cycle is 28 days; however, there is a large range both between and within individual women with a range of 25-32 days considered to be normal. The greatest variability in cycle length occurs in the years following the onset of menarche and proceeding menopause (Vollman, 1956). Menarche, the onset of menstrual bleeding and cyclicity, is one of the later stages of puberty in girls and the average age of onset is 12 years, however, factors such as genetics, diet, obesity and overall health can accelerate or delay the onset of menarche (Kaprio *et al.*, 1995). Menopause is the cessation of menstrual cycles at the end of a woman's reproductive life and the average age of menopause is around 50 years, although the exact age is influenced by a number of factors including genetics, chronic disease, certain surgeries and various medical treatments which can delay or hasten its onset (Kato *et al.*, 1998).

The sequence of events in the menstrual cycle is determined by the relative hormone concentrations at each stage of the cycle. Menstrual cycles are counted from the first day of menstruation and can be divided into 3 main phases or events; the follicular phase, ovulation and the luteal phase (Fig. 1-7). Each phase of the cycle corresponds with significant events in hormone concentrations, follicular development and endometrial pathology.

The follicular phase of the menstrual cycle spans the first day of menstruation until ovulation. The aim of the follicular phase is to develop a viable follicle capable of undergoing ovulation. The early events of the follicular phase are initiated by a rise in FSH concentrations, early in the cycle, which is caused by the decrease in progesterone and oestrogen concentrations at the end of the previous cycle, and the subsequent removal of inhibition of FSH secretion by these ovarian hormones (Sherman *et al.*, 1975). FSH stimulates the development of 15-20 follicles each month and stimulates follicular secretion of oestradiol by up-regulating secretion of androgens by the theca externa and by activating receptors on granulosa cells (Ganong, 2005). As oestradiol concentrations increase, under the influence of FSH, oestradiol inhibits the further secretion of FSH and FSH concentrations decrease (Abraham *et al.*, 1972; Sherman *et al.*, 1975).

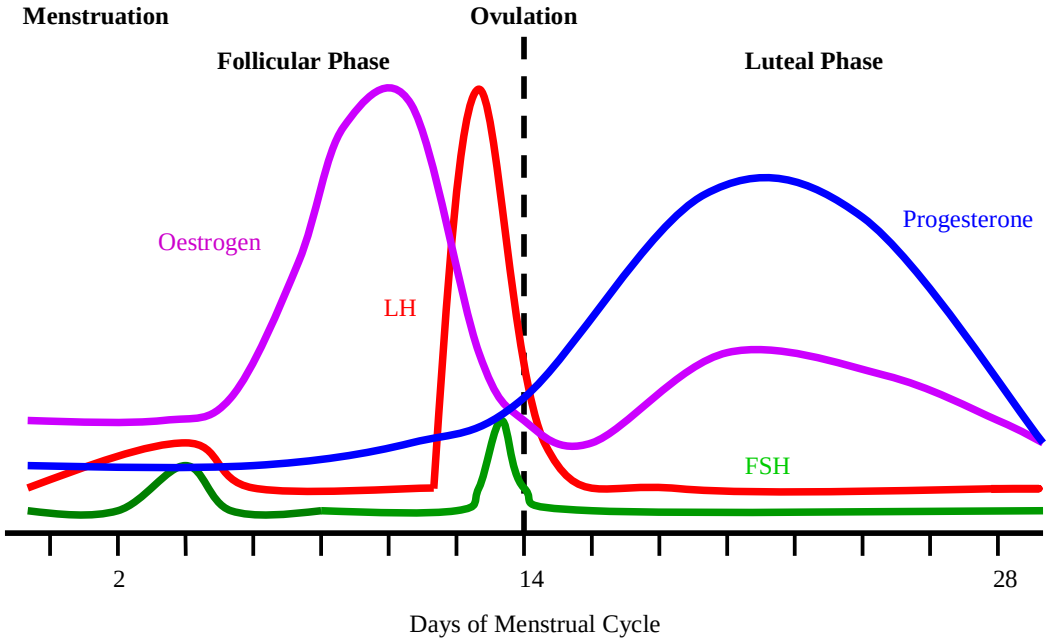

Figure 1-7. Schematic diagram showing the major hormonal events of the average menstrual cycle and the phases in which they occur.

Under normal circumstances, one follicle evolves into the dominant follicle, destined for ovulation, while the remaining follicles undergo atresia (van Santbrink *et al.*, 1995). The exact selection process of the dominant follicle has not been reported, however, it is known that the dominant follicle always expresses an abundance of FSH receptors (Hillier, 1994). As FSH concentrations decrease towards the end of the follicular phase, the developing follicles must compete for the remaining FSH and the dominant follicle, with its high concentration of FSH receptors, remains responsive (Hillier, 1994). The dominant follicle can then continue to synthesize oestradiol, which is essential for its complete maturation. The remaining, poorly FSH receptor-endowed follicles can not produce the required oestradiol, cease to develop and ultimately undergo atresia. As the dominant follicle matures, it secretes increasing amounts of oestrogen, the concentrations of which peak towards the end of the follicular phase (Abraham *et al.*, 1972). At this critical moment, oestrogen exerts positive feedback on GnRH and thus LH secretion, generating a dramatic preovulatory LH surge (Abraham *et al.*, 1972).

Menstruation occurs at the start of the follicular phase and is characterised by menstrual bleeding, during which the apoptosed functionalis portion of the endometrium is shed along with blood from the spiral arteries of the endometrium (Ferenczy et al., 1991). The increasing concentrations of oestrogen that occur during the follicular phase end menses

and induce proliferation of the new functionalis from stem cells of the basalis of the endometrium. Proliferation of endometrial glands and stromal connective tissue occurs towards the end of the follicular phase and continues through ovulation and into the luteal phase (Ferenczy et al., 1991).

The LH surge triggers ovulation by causing final maturation of the dominant follicle and weakening the wall of the follicle in the ovary (Chappel *et al.*, 1991; Hillier, 1994). The luteal phase is defined by the luteinization of the components of the follicle which were not ovulated and begins at ovulation and lasts until the menstrual phase of the next cycle. The granulosa cells, theca cells, and some surrounding connective tissue are all converted into the corpus luteum, which eventually undergoes atresia (Ganong, 2005). The major effects of the LH surge, apart from ovulation, are the conversion of granulosa cells from predominantly androgen-converting cells to predominantly progesterone-synthesizing cells, the expression of new LH receptors which fosters increased progesterone synthesis, and reduced affinity of granulosa cells for oestrogen and FSH (Ganong, 2005). Combined, these changes promote increased progesterone secretion with some oestrogen secretion (McNatty *et al.*, 1979; McNatty *et al.*, 1980). Progesterone secretion by the corpus luteum peaks between five and seven days post-ovulation (Abraham *et al.*, 1972). High progesterone concentrations exert negative feedback on GnRH and subsequently GnRH pulse frequency decreases. As GnRH pulse frequency decreases, FSH and LH secretion also decreases (Ganong, 2005). The corpus luteum further loses its FSH and LH receptors and, lacking stimulation by FSH and LH, after 14 days, the corpus luteum undergoes atresia and begins evolving into the corpus albicans (Ganong, 2005). With the decline of both oestrogen and progesterone concentrations, an important negative feedback control on FSH is removed and FSH concentrations rise once again to initiate the next menstrual cycle (Abraham *et al.*, 1972).

The menopause marks the end of reproductive capabilities of a woman and the end of menstrual cyclicity. The menopausal transition is considered to have been initiated when changes in cycle frequency or in menstrual flow first occur, and during this period, which lasts an average of 4 years, there are changes in the endocrinology of the pituitary-ovarian axis (McKinlay *et al.*, 1992). Up until the age of approximately 40 years there is a steady and roughly linear decline in follicle numbers within the ovary, however, over the following 10 years there is markedly accelerated decline in follicles, up until the menopause, where the ovary no longer contains follicles and therefore no longer contains granulosa cells (Burger, 1996). This sharp decline in follicles is thought to be linked to the rise in serum FSH concentrations that occur at this time, even in women who are

considered to be cycling regularly (Reyes *et al.*, 1977; Sherman *et al.*, 1975). Other than an overall average increase in FSH concentrations during the menopausal transition there are no other consistent hormonal changes with fluctuations in LH and oestrogen, as well as occasionally FSH being recorded by a number of studies (Hee *et al.*, 1993; Metcalf, 1988; Metcalf *et al.*, 1981). It is likely that these fluctuations are due to irregular maturation and ovulation of declining follicles.

The exact timing of true menopause and the cessation of menstrual periods in an individual can only be determined retrospectively, with no clear endocrine change occurring at the time of the last cycle (Burger, 1996). In the first 6-12 months following the last cycle, up to 40% of women have oestrogen concentrations consistent with functioning follicles; however, progesterone concentrations suggest that no ovulation takes place (Rannevik *et al.*, 1986). By 12-24 months after the menopause, serum oestradiol concentrations are known to be significantly lower than those pre-menopause (Rannevik *et al.*, 1986). Serum gonadotrophin concentrations increase significantly after the menopause and by 12 months post-menopause, serum FSH concentrations are 10-15 fold higher than follicular phase concentrations in young cycling women, whilst LH concentrations are approximately 3 fold higher (Burger, 1996; Chakravarti *et al.*, 1976). Serum gonadotrophin concentrations subsequently slowly decline with increasing age (Rossmanith, 1995).

1.12 Reproductive Hormones in the Bitch

Although there has been much debate as to the aetiology and pathophysiology of acquired urinary incontinence in the bitch it is accepted that neutering (either via ovariohysterectomy or ovariectomy) significantly increases the risk of a bitch developing this condition. This is thought to be due to changes in reproductive hormones that occur post neutering. To understand how these hormones may change after neutering, knowledge of the normal reproductive hormone patterns and the regulation of hormone secretion in the intact bitch is necessary.

The reproduction biology of the domestic bitch is unique. Unlike the more extensively studied species such as the rat and ovine, the bitch is usually classified as monoestrus with an inter-oestrus period of approximately 6 months regardless of pregnancy status. During the normal reproductive cycle of the bitch there are various known hormonal changes orchestrated by the hypothalamus and pituitary gland which in turn act on the ovaries; this is termed the hypothalamo-pituitary-ovarian axis. The bitch's oestrus cycle is commonly divided into 4 distinct stages, each with characteristic hormonal, behavioural and reproductive tract changes. These stages are pro-oestrus, oestrus, metoestrus and anoestrus (Fig. 1-8).

Pro-oestrus is associated with follicular development that is stimulated by Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), which in turn are controlled by Gonadotrophin Releasing Hormone (GnRH) released from the hypothalamus. This phase lasts for approximately 10 days. As the follicles develop they produce oestrogen in increasing quantity, which stimulates pheromone secretion and causes the bitch to become attractive to dogs, although she will not accept mounting (Concannon, 1986b). Although FSH is folliculotrophic it does not increase in concentration as dramatically as LH (Concannon, 1993), presumably because the developing follicles secrete inhibin, a selective inhibitor of FSH secretion (Olson *et al.*, 1982). The cells of the developing follicle begin to undergo luteinization before ovulation (Concannon *et al.*, 1977). This allows the cells to produce progesterone in low concentrations before ovulation, when oestradiol is the main follicular steroid, and it is understood to be this increase in progesterone and the fall in the oestrogen:progesterone ratio that triggers the LH and FSH surge and therefore ovulation (Concannon *et al.*, 1977; Concannon *et al.*, 1975; Olson *et al.*, 1982; Smith *et al.*, 1974). The LH surge is the central event in the oestrous cycle; it lasts for 1-3 days and occurs 0-3 days after the oestrogen peak (Concannon *et al.*, 1977).

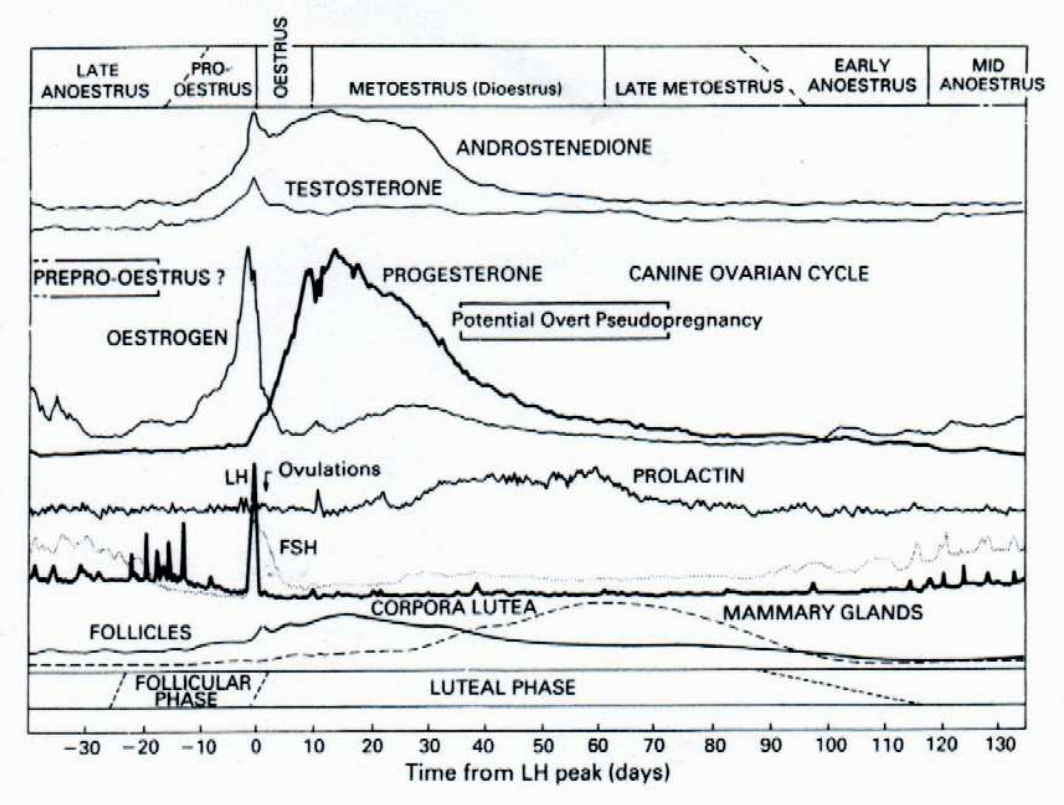

Figure 1-8. Schematic summary of hormonal changes considered typical of a non-pregnant ovarian cycle in a bitch. Taken from Canine pregnancy and parturition, Veterinary Clinics of North America: Small Animal Practice, volume 16:3, page 454, Copyright Elsevier (1986), used with permission.

Oestrus refers to the behavioural changes expressed by the bitch, namely the change from the proceptive behaviour of pre-oestrous, to receptive behaviour (Beach *et al.*, 1982). The exact timing of the onset of oestrus when compared to the LH peak varies between individual bitches but tends to be within one day of the LH surge (Concannon *et al.*, 1989). The rapidly decreasing oestrogen concentrations in the presence of the rising progesterone concentrations bring about these behavioural changes (Concannon *et al.*, 1977). The LH surge causes ovulation and further luteinization of the ruptured follicles to become corpora lutea, allowing the cells to switch from producing oestrogen to progesterone (Concannon *et al.*, 1977).

Metoestrus is deemed to have commenced when a bitch refuses to stand to be mated and is usually 6-8 days after the onset of oestrus, or 8-10 days after the LH peak (Jeffcoate, 1998). There are no discrete endocrine changes that mark the onset of metoestrus, although the plasma progesterone will have been increasing since just prior to the pre-ovulatory LH peak and will continue to rise. This rise in progesterone is considered a useful indicator of ovulation, and plasma progesterone concentrations of around 10ng/ml

clearly coincide with the most fertile period in the bitch, about 3-5 days after the LH peak (Jeffcoate *et al.*, 1997). The progesterone concentration reaches a plateau approximately 2 weeks after the LH peak (Concannon, 1983; Weilenmann *et al.*, 1993) after which concentrations of progesterone gradually decline over the next 60-120 days (Austad *et al.*, 1976). This decline is thought to be due to luteal regression, which is poorly understood in the canine. No luteolytic mechanisms have been found in the bitch (Hoffmann *et al.*, 2004), and it is postulated that regression could be due to ageing of the corpora lutea. In the bitch, progesterone is produced by the corpora lutea, progesterone production is supported in the first half of the luteal period by LH (Concannon, 1980; Okkens *et al.*, 1986) but prolactin is the predominant luteotrophic factor in the second half of the luteal period (Concannon *et al.*, 1987; Jochle *et al.*, 1989; Okkens *et al.*, 1986). Prolactin concentrations typically rise from approximately day 30 to day 65 in non-pregnant bitches and then begin to fall (Concannon, 1993; Jeffcoate, 1998). Oestradiol concentrations increase very slightly near the start of metoestrus and remain at this level during the luteal phase, falling again towards the start of anoestrus (Onclin *et al.*, 2002).

Anoestrus is defined as the interval between the end of the luteal phase and the start of pro-oestrus. Determining the end of the luteal phase is difficult, as it is not marked by any obvious hormonal or behavioural changes. Progesterone concentrations tend to be lower than 1ng/ml at the start of anoestrus (Jeffcoate, 1998) and remain low throughout anoestrus (Concannon, 1986b; Weilenmann *et al.*, 1993). FSH, in contrast, is high throughout anoestrus (Olson *et al.*, 1982) and there is evidence that FSH concentrations increase towards the end of anoestrus and that this increase in circulating FSH should be considered a critical event required for ovarian folliculogenesis (Kooistra *et al.*, 2001). LH concentrations are on average low throughout most of the cycle, except during the pre-ovulatory LH surge (Concannon *et al.*, 1977; Concannon, 1993). It has now been shown that LH secretion is pulsatile throughout the entire cycle (Hegstad *et al.*, 1993; Hoffmann *et al.*, 1993; Jeffcoate, 1993) and that pulse frequency increases during anoestrus to a maximum in late anoestrus and then decreases in pro-oestrus, although mean concentrations in plasma were high during proestrus (Concannon, 1993). During the transition into anoestrus oestradiol concentrations are still raised above basal but fall to basal concentrations before rising considerably approximately a month before the LH peak (Jeffcoate, 1993). Prolactin concentrations fall in early anoestrus and increase slightly during pro-oestrus although the reasons for this remain unclear (Jeffcoate, 1993).

There has been much interest in the mechanism that maintains such a long inter-oestrus period in the bitch. Unlike many other domestic species there appears to be no dramatic

effect of photoperiod (Bouchard *et al.*, 1991). The high concentrations of sex steroids during metoestrus exert a negative feedback control on the hypothalamus and pituitary suppressing FSH and LH production, however, as the concentrations of the sex steroids wane towards anoestrus this negative feedback is removed and FSH and LH production increases. The high FSH and pulsatile LH seen throughout anoestrus suggest that there is no lack of gonadotrophin production so research has concentrated on an ovarian insensitivity mechanism to explain the lack of follicular activity during anoestrus. To date the best explanations seem to be an effect of luteal remnants on follicular gonadotrophin sensitivity (Jeffcoate, 1993) or follicular insensitivity to FSH caused by FSH receptor splice variants found at this stage of the cycle which may prevent the follicular response to FSH stimulation (McBride *et al.*, 2001).

The main effect of neutering a bitch is the irreversible termination of her reproductive cyclicity, with a neutered bitch staying in what is often considered to be a permanent state of anoestrus. This is caused by the lack of oestrogen and progesterone production due to the removal of her ovaries (Concannon, 1993). It also is known that neutering leads to a rapid and persistent increase in the circulating plasma concentrations of LH and FSH, due to the lack of negative feedback on the pituitary and hypothalamus by gonadal steroids (Burger, 1996; Olson *et al.*, 1992; Reichler *et al.*, 2004).

1.12.1 The Role of Reproductive Hormones in Urinary Incontinence in the Bitch

Initially, the development of acquired urinary incontinence post-neutering in the bitch was thought to be caused by the lack of oestrogen (Finco *et al.*, 1974), and treatment with supplemental exogenous oestriol is still a current pharmacological treatment for acquired urinary incontinence in the bitch (Angioletti *et al.*, 2004). Despite this, it is unlikely that oestrogen deficiency alone is responsible for acquired urinary incontinence as oestrogen replacement therapy is ineffective in about 25% of cases (Arnold *et al.*, 1989) and the serum oestradiol concentration of spayed incontinent bitches is similar to that of intact bitches during anoestrus (Richter *et al.*, 1985). Due to this, a new hypothesis has been formed that the increase in gonadotrophins may be at least partially responsible for the development of acquired urinary incontinence, especially as studies have demonstrated the presence of receptors for the gonadotrophins in the urinary bladder (Ponglowhapan *et al.*, 2007a; Reichler *et al.*, 2007; Tao *et al.*, 1998). Further support for this indirect effect of

steroid removal is provided by a recent study in neutered canines suffering from acquired urinary incontinence which reported that administration of GnRH analogues to decrease serum LH and FSH concentrations aided continence (Reichler *et al.*, 2006b). If increased concentrations of gonadotrophins are involved in the development of acquired urinary incontinence then the success of replacement oestrogen therapy in the bitch may be partly due to the suppression of plasma LH and FSH by oestrogen administration (Concannon, 1993).

1.13 An Overview of the Treatment Options for Urinary Incontinence

The aim of treatment of urinary incontinence is curing or lessening the clinical signs of overt inappropriate urination, preventing complications and secondary infections, and improving the quality of life for the patient. Treatment of urinary incontinence can be either pharmacological or non-pharmacological. Non-pharmacological measures include increased scheduled opportunities to void, bladder training, pelvic floor exercises, biofeedback / electrical stimulation, periurethral injection therapy, surgery, catheters and incontinence pads, although not all measures are suitable for both women and bitches.

As many patients afflicted by urinary incontinence, both human and canine, suffer from episodic incontinence, shortening the inter-voiding time can help to decrease the number of episodes of incontinence. This can be especially useful in canines who suffer most from inappropriate urination overnight where the inter-voiding interval can be as long as 10 hours. Scheduled toileting and bladder training are completely non-invasive management options suitable mainly for use in women where the patient practices voiding on a schedule and consciously suppresses sensory urges to urinate in an attempt to train the bladder and brain to coordinate urination, as occurs with initial toilet training (Holroyd-Leduc *et al.*, 2004b). This can be effective in a number of cases of mild urinary incontinence; however, it does require good cognitive function on behalf of the patient (Moore, 2000; Thakar *et al.*, 2000).

Pelvic floor exercises are a mainstay of treatment for urinary incontinence in women and are effective in strengthening the pelvic floor muscles, and this is particularly important in women with stress incontinence, but again is not a feasible option in canines with acquired urinary incontinence. These exercises help increase a woman's awareness of her pelvic muscles and studies suggest they can increase the strength and duration of pelvic muscle contractions, decrease the volume of urine leakage and decrease the number of incontinent episodes a day (Thakar *et al.*, 2000). Vaginal cones and weights can also be inserted into the vagina as a means to strengthen pelvic floor muscles and are often recommended as an adjunct to pelvic floor exercises (Holroyd-Leduc *et al.*, 2004b; Thakar *et al.*, 2000).

Electrical stimulation therapy involves the electrical stimulation of pelvic floor muscles in women using either a probe wired to a device for controlling the electrical stimulation, or extracorporeal pulsed magnetic innervation. It is thought that pelvic floor stimulation of

the pudendal nerve will improve urethral closure by activating the pelvic floor musculature (Holroyd-Leduc *et al.*, 2004a). In addition, electrical stimulation is thought to improve partially denervated urethral and pelvic floor musculature by enhancing the process of reinnervation. Variation in the amplitude and frequency of the electrical pulse is used to mimic and stimulate the different physiologic mechanisms of the voiding response, depending on the type and aetiology of incontinence, e.g., either overactive bladder, stress incontinence, or a mixed pattern, although best results seem to obtained with patients suffering from overactive bladder incontinence (Brubaker *et al.*, 1997; Wang *et al.*, 2004).

Periurethral injection therapy has been used successfully to treat urinary incontinence in both women and bitches (Barth *et al.*, 2005; Thakar *et al.*, 2000). In this procedure an inert sclerosing agent such as gluteraldehyde cross-linked bovine collagen is injected into the urethra to physically decrease the urethral lumen. The procedure is repeatable if required and carries a high success rate coupled with a low complication rate (Barth *et al.*, 2005; Thakar *et al.*, 2000). Although the procedure is commonly practiced in human medicine it is not widely available in veterinary practice, with only a handful of veterinary hospitals offering the treatment in America and mainland Europe, and none offering the procedure in Britain, as yet.

Colposuspension is an established surgical technique for the treatment of stress incontinence in women and urethral sphincter mechanism incompetence in the bitch (Holroyd-Leduc *et al.*, 2004a; Holt, 1990). In both species, the surgery is similar and involves placing non absorbable sutures from the bladder and cranial vagina to the body wall at the level of the pubic bone to draw the bladder neck out of the pelvic inlet and into the abdomen. This means that any increases in intra-abdominal pressure can act simultaneously on the bladder and urethra. Thus, any increase in intravesical pressure is counteracted by an increase in urethral resistance (Holt, 1990). Although the procedure claims to have a high long-term success rate and low complication rate, it is a very invasive procedure and careful evaluation of the patient is required prior to surgery so that only those patients with genuine stress incontinence and an intrapelvic bladder are selected, as these are the patients that respond most favourably to this treatment (Holroyd-Leduc *et al.*, 2004a; Holt, 1990). In women, a laparoscopic colposuspension technique has been reported that is less invasive and gives shorter recovery times, however it appears to be 20% less effective than open surgery (Thakar *et al.*, 2000).

Tension free vaginal tape is a surgical technique for the treatment of genuine stress incontinence in women. In it, a special prolene tape is inserted via suprapubic incisions

through the urogenital diaphragm to pass laterally to the urethra and behind the pubic bone to perforate the rectus sheath. This means that the tape comes to rest in a U shape around the mid-urethra and is then adjusted so that it provides tension free support to the urethra. Good success rates have been recorded with improvement in up to 95% of patients, at least in short term studies (Moran *et al.*, 2000; Ulmsten *et al.*, 1999). Due to the ease of the surgical technique, a relatively low incidence of serious complications and the short post-operation recovery time, this procedure has increased in popularity and is considered an excellent alternative to colposuspension in many patients (Thakar *et al.*, 2000). This procedure has been described in the bitch (Nickel *et al.*, 1998), however, the number of complications, coupled with a relatively poor success rate when used alone, means that this procedure is not in common use in the bitch.

There are several other surgical procedures documented for treatment of urinary incontinence including urethral reconstruction, ileocytoplasty and artificial urinary sphincter placement (Thakar *et al.*, 2000). Although none of these are routine procedures, artificial urinary sphincter placement is indicated in a number of women such as those with sphincteric dysfunction in which other procedures have not significantly improved the condition. These are not procedures that are utilised in the canine (Elliott *et al.*, 1998; Petrou, 2002).

As previously mentioned there are a number of pharmacological treatments for urinary incontinence in both the woman and the bitch. In the woman there are specific therapies suitable for treating urinary incontinence due to an overactive bladder, and separate treatments for stress incontinence which concentrate on treating urethral dysfunction. In the bitch the only reported and licensed therapies are for treating urinary incontinence due to decreased resting urethral tone. In both species it is often found, however, that combination therapy, especially involving a mix of medical, behavioural and non-specific treatments gives the best and most sustainable cure for the patient.

In the bitch the most commonly used pharmacological treatments in the United Kingdom are oestrogens (Incurin™, Invervet) and alpha-adrenergic drugs, the most common being phenylpropanolamine (Propalin™, Vétoquinol). They both act by increasing the resting urethral tone, therefore are of limited use in patients with incontinence due to causes other than sphincteric dysfunction. Both drugs seem to be well tolerated by animals, however, there is a risk of side effects with both drugs, the main symptoms being increased attractiveness to male dogs and increased risk of mammary cancer with oestrogens (Hotston Moore, 2001; Mandigers *et al.*, 2001) and increased risk of aggressiveness and

increased blood pressure with phenylpropanolamine (Carofiglio *et al.*, 2006; Mandigers *et al.*, 2001). Although widely used, these treatments do not always affect a cure and many bitches become refractory to them over time (Mandigers *et al.*, 2001; Richter *et al.*, 1985). Recently, a long acting GnRH analogue has been trialled in the bitch as a treatment for acquired urinary incontinence and it appears, at least in the short term, to offer good clinical improvement in urinary incontinence in this species, although the exact mechanism of action has not been described (Reichler *et al.*, 2006b).

In women, the mainstay of medical management for stress incontinence has also been hormonal treatment with oestrogen and progestins and with alpha-agonists such as phenylpropanolamine, however, side effects can be great and these are no longer recommended for long term treatment of urinary incontinence. Oestrogens were used for stress incontinence, as weakened pelvic floor muscles due to decreased concentrations of oestrogen post-menopause are considered one of the main contributing factors to development of urinary incontinence. Oestrogen has been shown to have a direct effect on urethral mucosa and periurethral tissues, increasing urethral closure pressure (Sarkar *et al.*, 2000); however, side effects of long term use include significantly increased risk of breast cancer, ovarian cancer, cholecystitis, cardiovascular disease and stroke (Chen *et al.*, 2002b; Grady *et al.*, 2002; Hulley *et al.*, 2002; Lacey *et al.*, 2002; Nelson *et al.*, 2002). This means that the long term risks of systemic treatment with oestrogen in women outweigh the advantages of treatment and it is no longer a recommended therapy. Topical oestrogen vaginal creams, the oestrogen vaginal ring and oestrogen patches are associated with few side effects, however, and appear to be as effective as systemic treatment and therefore are recommended for a number of patients (Robinson *et al.*, 2003).

Other therapies for stress incontinence in women include the α-adrenoceptor agonists phenylpropanolamine and pseudoephedrine and also the selective serotonin and noradrenalin reuptake inhibitor duloxetine. Phenylpropanolamine has recently been withdrawn from the market due to a slight but significant increase in the risk of haemorrhagic stroke associated with its use (Norton *et al.*, 2006). Pseudoephredine is still available and works by increasing the urethral sphincter tone, however, it also acts upon other body systems to give side effects such as insomnia, restlessness, nervousness and headaches and can cause fatal complications in patients suffering from cardiac arrhythmias, hypertension and angina and therefore has limited applications (Norton *et al.*, 2006). Duloxetine is a relatively new therapy that has undergone a number of clinical trials in which it has shown good efficacy in treating stress incontinence in women with a low risk of significant side effects, although a number of patients complain of dry mouth

and nausea (Hurley *et al.*, 2006). Although new therapies are coming onto the market to treat stress incontinence in women it is likely that behavioural approaches and pelvic floor exercises are just as effective in treating stress incontinence in the majority of women as pharmacological agents.

Pharmacological treatments for urge incontinence caused by an overactive bladder in the woman are more promising and offer an improved benefits/risk ratio than those for stress incontinence. Anticholinergic and antispasmodic agents such as oxybutynin and tolterodine are the most common choices for the treatment of urge incontinence and act by decreasing muscarinic-mediated detrusor contractions and allowing the bladder to fill properly. Oxybutynin is an antimuscarinic agent and also has a direct antispasmodic effect on smooth muscle whereas tolterodine is purely a muscarinic receptor antagonist. Both agents are effective at improving continence and quality of life for patients (Diokno *et al.*, 2003; Norton *et al.*, 2006). Side effects of both drugs are primarily anticholinergic and dose related and include xerostomia, xerophthalmia, constipation, sedation, blurred vision, urinary retention, insomnia, tachycardia, confusion and delirium. A large number of studies, many associated with drug companies, have looked at the effects of immediate and sustained release oxybutynin and tolterodine and have produced many contradictory claims. In general, it has been concluded that immediate release oxybutynin is as effective as immediate release tolterodine, however, the latter has the least incidence of dry mouth due to the fact that it is more selective for receptors in the bladder than in the parotid gland (Harvey *et al.*, 2001; Malone-Lee *et al.*, 2001). The results for the sustained release preparations of oxybutynin and tolterodine have shown that both agents work well, with oxybutynin being slightly more effective in increasing the number of patients with no clinical signs of incontinence at 12 weeks, however, oxybutynin was also reported to produce a higher incidence of dry mouth (Diokno *et al.*, 2003). To further reduce the anticholinergic side effects of these drugs a transdermal oxybutynin product has been trialled and manufactured which appears to have similar efficacy to oral immediate-release oxybutynin but with lower anticholinergic side effects, however, the cost of this treatment is significantly more than oral therapies and there are reported application site reactions with this mode of delivery (Davila, 2006; Davila *et al.*, 2001; Dmochowski *et al.*, 2002). There have been no reported trials of muscarinic receptor antagonists for urinary incontinence in the bitch therefore efficacy and safety of these drugs in canines is at present unknown.

Tricyclic antidepressants can also be used for urge incontinence in women. These agents facilitate urine storage by increasing outflow resistance and by decreasing bladder

contractility (Yoshimura *et al.*, 2002). The tricyclics that have been used include nortriptyline, desipramine, imipramine, doxepin and amitriptyline, however, the latter should be avoided in elderly or frail patients as it is associated with increased anticholinergic effects than the other tricyclics (Yoshimura *et al.*, 2002). There are no reported studies looking at the use of these drugs in the bitch, however amitriptyline has been used for behaviour modification therapy and is known to have severe cardiac effects including causing ventricular tachycardia in some animals (Ansel *et al.*, 1993). These agents are likely best used when the patient has a concomitant depression or neuropathy.

In conclusion there are many treatment options for urinary incontinence in both women and bitches, however, not all treatments are suitable for all patients, therefore, a careful and thorough work-up leading to accurate diagnosis of the condition is required, along with a general clinical exam to diagnose any other existing medical conditions, before a specific treatment option is recommended. In most patients a combination of therapies will give the best outcome, with behavioural therapy and modifications to lifestyle being the first step in formulating an individual treatment plan. Due to the potential side effects of both surgery and medical treatments these options should only be considered after less invasive therapies have been exhausted. In the bitch, behavioural therapy will often not result in a complete cure of the disease, therefore medical therapy should be considered alongside modifications to lifestyle. In both women and bitches surgery should only be offered after other treatments have failed and only after a thorough counselling of patient or owner as to the potential side effects and complications of the procedure. In all cases patients and owners should be warned that with current therapeutic options the condition may not be totally resolved despite a multi-modal treatment regime, in which case the use of incontinence pads and other sanitary devices may be required.

1.14 Conclusions and Aims

It is possible to see that there are many similarities between post neutering urinary incontinence in the bitch and stress incontinence in the woman, with both conditions centring on a decreased resting urethral tone that is insufficient to overcome increases in pressure within the bladder. It has also been shown that the bladder has an important role to play in the development of urinary incontinence in the woman, especially in cases of urge incontinence caused by an overactive bladder, and in cases of decreased bladder contractility. In the bitch the role of the bladder in the development of urinary incontinence has not been reported, although it is understood that a decreased resting urethral tone is not a defining characteristic of acquired urinary incontinence in the bitch (Reichler *et al.*, 2006b). This has lead to the hypothesis that the bladder may be involved in the development of urinary incontinence in the bitch and as such could provide a new target for treatment of this debilitating and embarrassing problem.

The aim of the studies in this thesis therefore, were to investigate the functional, structural and molecular changes that occur within the bladder of a canine post neutering that may lead it to develop, or predispose it to, urinary incontinence.

2 Validation of Equipment and Protocols

2.1 Introduction

The ultimate aim of this project was to determine and characterise the changes that occur within the urinary bladder of canines post neutering that may predispose a bitch to develop acquired urinary incontinence. As there have been no published *in vitro* reports of canine detrusor muscle function, the aim of this preliminary study was to validate our *in vitro* organ bath equipment, experimental technique and protocols by replicating the robust experimental approach previously described for use in the rat (Rattus rattus) (Kories et al., 2003; Longhurst et al., 2000). Using this approach, strips of rat urinary bladder are placed in organ baths under standard physiological conditions, tensioned until stable at the designated optimal resting tension (2g), and then subjected to standard concentration dependent carbachol and KCl response protocols. Carbachol is a non-selective muscarinic agonist that causes contraction of smooth muscle preparations via stimulation of the muscarinic pathway whilst KCl causes contraction of smooth muscle preparations by direct action on the cell membrane causing depolarisation. As contractile responses of urinary bladder strips in this experimental set up have proven to be consistent when used by a number of different research groups, similar responses in this study would validate our equipment and demonstrate reliable and consistent technique by personnel.

2.2 Materials and Methods

2.2.1 Animals

Age matched adult male (n = 10) Wistar rats (200 - 300g, out bred) obtained from Harlan (Oxfordshire, UK) were used for this study. All animals received food and water *ad libitum*. The protocols used for this investigation were approved by the Glasgow University Veterinary School Ethics Committee.

2.2.2 Preparation of Tissue

Rats were humanely killed through stunning followed by exsanguination by severance of the carotid arteries, in accordance with the Animals (Scientific Procedures) Act 1986. The urinary bladder was immediately removed and placed in ice-cold Krebs buffer of the following composition: NaCl 118mM, KCl 4.8mM, $CaCl_2$ 2.5mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $NaHCO_3$ 24mM, glucose 11mM. The dome of the bladder was dissected free at the level of the ureteral orifices, cleared of fat and cut into strips (2mm x 10mm).

The bladder strips were mounted in 15ml tissue baths (Scotia Glassware Ltd, Grangemouth, UK), bathed at 37°C in Krebs solution and continually aerated with 95% O_2 / 5% CO_2. Muscle tension was measured with BIOPAC TSD125C isometric force microtransducers (BIOPAC Systems Inc, California) and results displayed on a computer screen using the BIOPAC data acquisition software (AcqKnowledge 3.8) (Fig. 2-1). Bladder strips were mounted under 2g resting tension (Longhurst *et al.*, 2000) and the contractile response to KCl (40mM) was examined to determine tissue viability. Strips that did not produce a minimum of 0.5g active tension were discarded, replaced and new strips tested. After testing all strips were repeatedly washed and allowed to equilibrate for 30 minutes, during which time the resting tension was adjusted to 2g every 10 minutes.

In all cases experiments were performed on the same day as the bladders were harvested.

2.2.3 Carbachol Concentration Response Protocol

Once muscle tension in the strips was stable at 2g resting tension a standard Carbamylcholine Chloride (carbachol, Sigma, UK) concentration response protocol was performed and the resultant muscle tensions recorded. Briefly, carbachol was made up to a 1M stock solution and aliquots stored (-20°C) from which serial dilutions were prepared on the day of each experiment. Carbachol was added to the baths in a stepwise manner to give cumulative bath concentrations of 1nM – 30µM (log -9M to -4.5M), increasing in half log increments after the response to the previous concentration had reached a maximum.

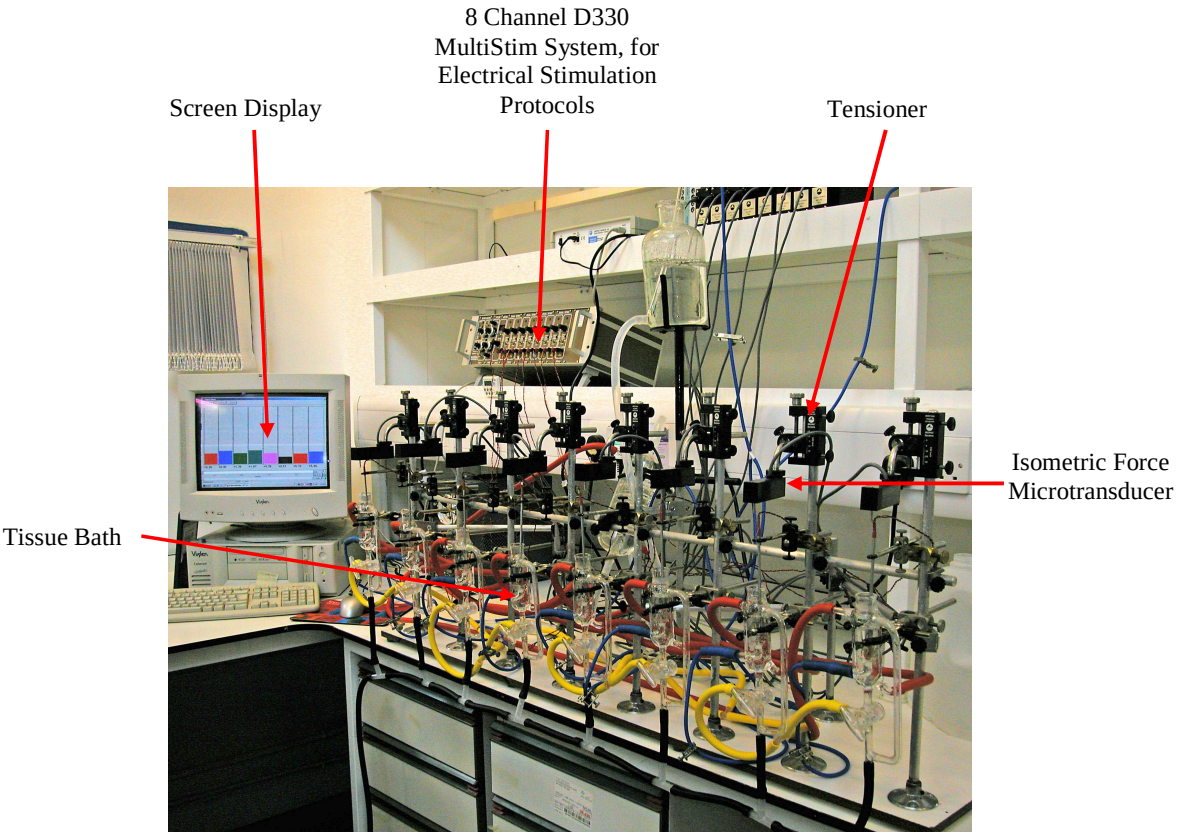

Figure 2-1. Photograph of the tissue bath and electrical stimulator set up.

2.2.4 KCl Concentration Response Protocol

Following completion of the cumulative response protocol to carbachol, the strips were washed repeatedly and allowed to equilibrate for 1 hour, during which time the resting tension was adjusted to 2g every 10 minutes. Once tension in the strips of urinary bladder were stable at 2g resting tension, KCl (Sigma, UK) (2M) was added to the baths in a stepwise manner to give final cumulative bath concentrations of 10mM – 80mM. This was performed using 10mM increments with each further concentration applied after the response to the previous concentration had reached a maximum.

2.2.5 Electrical Field Stimulation Protocol

In a separate series of experiments, strips of urinary bladder were mounted in Ag-AgCl ring electrodes (manufactured by Mr I. Gibson, Glasgow University Veterinary School), washed repeatedly and tensioned until stable at 2g resting tone. Once stable the supramaximal voltage (at 20Hz) was determined for each strip by applying stimulations at increasing voltages until the maximal contractile response was seen, the voltage required to produce approximately 70% of this response was considered the supramaximal voltage of that strip. Any strips that did not respond at this stage were discarded, replaced and the supramaximal voltage determined as before. A train of three stimulations at the supramaximal voltage for each strip was then conducted; to ensure that the response seen to that voltage was stable.

Bladder strips were then washed, re-tensioned to 2g resting tension and allowed to equilibrate for 30 minutes, during which time the strips were re-tensioned to 2g every 10 minutes. Once muscle tension was stable, electrical field stimulation (0.5-100Hz, 100 pulses) was delivered from a Digitimer Ltd MultiStim System-D330 stimulator (Hertfordshire, UK) at a pulse width of 0.5ms and at supramaximal voltage. Stimulations were applied at five minute intervals to allow the re-establishment of normal resting tone between stimulations. Frequency dependent contractions were observed. To allow confirmation that the observed responses to electrical field stimulation were neurogenic, at the end of the each experiment the tissue strips were incubated with the neurotoxin Tetrodotoxin (1µM) (Sigma, UK) and stimulated at 20Hz every 5 minutes for 3 stimulations. Tetrodotoxin is a neurotoxin that prevents action potentials forming and

spreading through a muscle by blocking the sodium gated voltage channels on nerve cells thereby preventing any nerve mediated contractions (Narahashi, 1974).

2.2.6 Data Analysis

All data are expressed as g/mg of wet tissue and results as mean ± s.e.mean where applicable (n = number of animals).

The results from all strips collected from each animal (minimum 4 / animal) were meaned, and the value obtained used as the result for that animal in further analysis. The $LogEC_{50}$ values (log of the concentration of carbachol required to produce a response equal to half of the maximal tension produced) were calculated by GraphPad Prism® v.5 software from the mean data for each animal.

2.3 Results

Carbachol induced concentration dependent contraction of the isolated urinary bladder strips in all animals (Fig. 2-2a). The threshold for response was approximately 30nM and maximum response was observed at approximately 10µM.

KCl induced a dose dependent contraction of all isolated urinary bladder strips (Fig. 2-2b), the maximal contraction produced was lower (37%) than that produced by carbachol. The threshold for response was approximately 20mM and maximum response was observed at approximately 50mM.

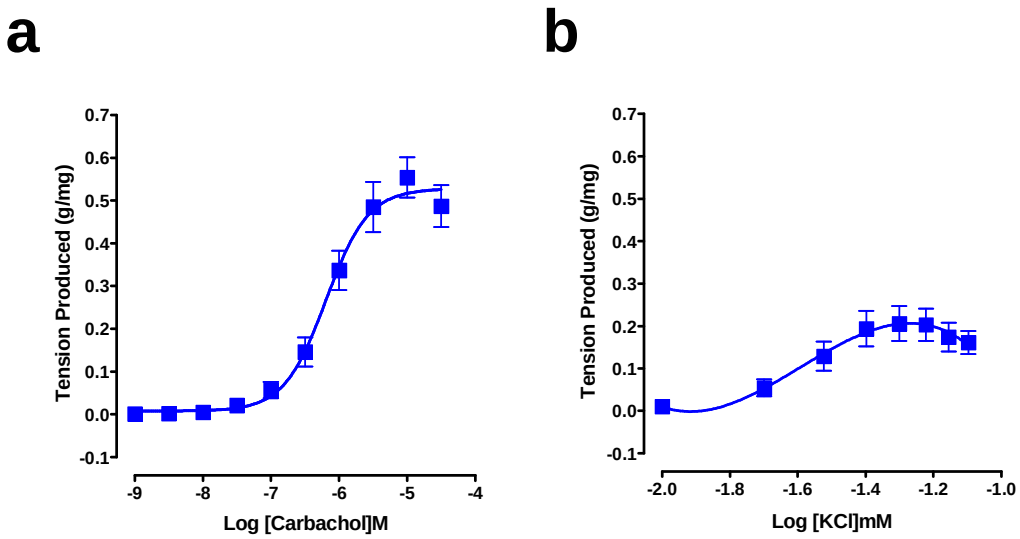

Figure 2-2. Cumulative dose response curves to a, carbachol and b, KCl in isolated rat urinary bladder strips. Each point is the mean ± s.e.mean of observations from 10 animals.

Electrical field stimulation of the isolated strips of urinary bladder produced frequency dependent contraction (Fig. 2-3). The maximal contraction was seen at 20Hz in all animals. Tetrodotoxin abolished the response of the tissue to electrical field stimulation and thus confirmed that responses seen were neurogenic in origin. The effects of tetrodotoxin are shown in the data record for a representative strip in Figure 2-4.

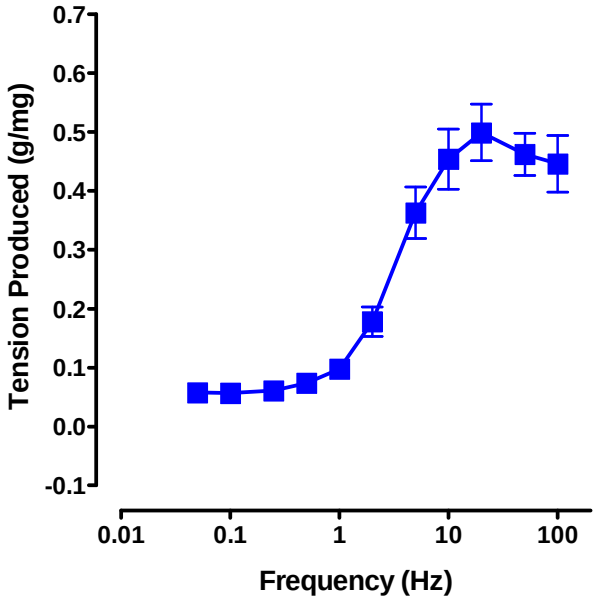

Figure 2-3. Frequency response curves in isolated strips of rat bladder. Each point is the mean ± s.e.mean of observations from 10 animals.

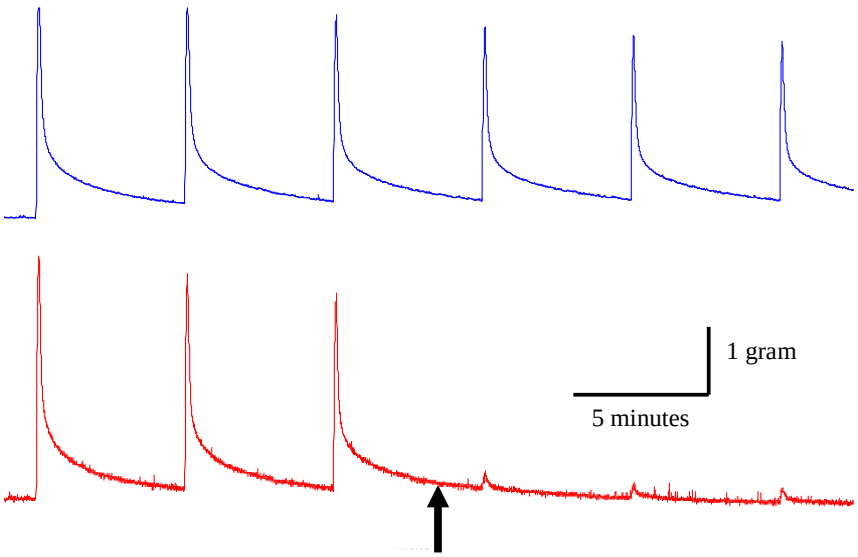

Figure 2-4. A typical section of trace showing control stimulations (upper trace) and the inhibitory effect of tetrodotoxin (1μM; lower trace, addition denoted by arrow) in isolated strips of rat urinary bladder. Stimulation parameters were 20Hz, 5 sec, and 0.5msec pulse width.

2.4 Discussion and Conclusion

In this study, the strips of rat urinary bladder were found to respond in a similar manner and with a similar magnitude of response to that reported previously, thus validating both our experimental tissue bath set-up and the methodology as suitable for the study of rat urinary bladder (Frazier *et al.*, 2007; Kories *et al.*, 2003; Longhurst *et al.*, 2000), and therefore reasonable for the study of canine detrusor muscle function.

There was concentration dependant contraction of the rat urinary bladder strips to carbachol. This is as expected as carbachol is a non-selective muscarinic agonist that works via the cholinergic system, binding to and activating the muscarinic receptors within the bladder (Rang *et al.*, 2007). As the muscarinic receptors are known to mediate the contractile response of the urinary bladder (Longhurst *et al.*, 2001) then addition of carbachol can be expected to produce contraction in a concentration dependant manner. Although carbachol acts at the muscarinic receptors by mimicking acetylcholine, it is not hydrolysed by acetylcholinesterase and therefore is not broken down or removed (Rang *et al.*, 2007), hence the contraction produced by each dose of carbachol does not diminish but remains stable over time. Carbachol is thought to act mainly at the M_3 receptors in the bladder, which are the receptors deemed responsible for the majority of the contractile action of the bladder during micturition (Chess-Williams, 2002); however, it is possible that at higher concentrations carbachol may act on the M_2 and nicotinic receptors causing a change of state of the cation channels on the muscle cell membrane (Andersson *et al.*, 2004a; Rang *et al.*, 2007), and potentially altering both K^+ and Ca^{2+} conductance across the cell membrane. The change in state of K^+ ion channels, in particular, may explain why at higher concentrations, carbachol was seen to invoke concentration dependant relaxation of the rat urinary bladder strips in this present study (Oh *et al.*, 2003).

In response to KCl, there was concentration dependant contraction, followed by concentration dependant relaxation of the rat urinary bladder strips. KCl is a metal halide that disassociates in solution to form K^+ (the major cation of cells) and Cl^- ions, both of which are essential to many body systems and functions including conductance of nerve impulses, acid-base balance and carbohydrate metabolism. KCl is not specific for any receptor but is thought to act directly upon the cell membrane (Foster *et al.*, 1983). When added to the extracellular environment KCl, at low concentrations, causes depolarisation of the cell membrane thereby leading to formation of action potentials and contraction of smooth muscle cells. At higher concentrations it is possible that alterations in relative

concentrations of K^+, Ca^{2+}, Na^+ and Mg^{2+} ions may cause the hyperpolarisation of the cell membrane and activation of various ion channels thereby inhibiting further contraction (Foster *et al.*, 1983; Kravtsov *et al.*, 1995), and eventually leading to relaxation.

In response to electrical field stimulation, there was frequency dependant contraction of the urinary bladder strips which was blocked by the neurotoxin tetrodotoxin. Tetrodotoxin is a neurotoxin that prevents action potentials forming and spreading through a muscle by blocking the sodium gated voltage channels on nerve cells thereby preventing any nerve mediated contractions (Narahashi, 1974). Using this neurotoxin demonstrated that the responses obtained in the experiments previously described were neurogenic in origin, therefore, they mimicked the *in vivo* contractile responses to parasympathetic neuronal stimulation of the bladder during the emptying phase of micturition. This contraction during the emptying phase is thought to be mediated mainly through muscarinic receptor stimulation (Andersson *et al.*, 2004a; Andersson *et al.*, 2004b; Creed *et al.*, 1983; D'Agostino *et al.*, 1989) via acetylcholine released from the nerve terminals. As free acetylcholine around the nerve terminal is rapidly broken down by the enzyme acetylecholinesterase (Andersson *et al.*, 2004a) the contraction evoked by electrical field stimulation cannot be maintained over time, hence the need to allow sufficient time for the tissue to recover between stimulations.

The responses of the rat bladder to carbachol, KCl and electrical field stimulation showed little variation both between strips for an individual animal and between individual animals. This was to be expected as the study population was uniform in terms of weight, age and strain of animal used, and confirms that error due to personnel or equipment was minimal. These results confirm that the techniques and equipment are suitable to investigate changes in bladder function in the canine, and demonstrate that any differences in function of detrusor muscle recorded in the canine are not due to personnel or equipment error.

3 Validation of Canine Tissue Bath Protocols

3.1 Introduction

Acquired urinary incontinence in the bitch is an increasingly recognised condition that affects up to 20% of all neutered bitches (Arnold *et al.*, 1989). The role of the urethra in the condition has been extensively studied (Gregory, 1994; Holt, 1988; Reichler *et al.*, 2004; Rosin *et al.*, 1981), however, decreased urethral tone is not considered a defining characteristic and the exact aetiology and pathophysiology of the condition remain unknown. Studies in women, to investigate post menopausal urinary incontinence, have demonstrated that the bladder has a pivotal role to play in the development of urinary incontinence in this patient group (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b; Resnick *et al.*, 1987). The overall aim of this thesis was to determine and characterise the changes that are induced within the urinary bladder of canines post neutering that may predispose them to develop acquired urinary incontinence. As there are no published *in vitro* reports of canine detrusor muscle function, the aim of this part of the study was to validate protocols for use with this tissue within a classical *in vitro* tissue bath system.

The equipment and protocols to be used with the canine urinary bladder tissue had previously been validated using bladder tissue from a species in which similar studies have been conducted, namely the rat, (chapter 2). The results in the rat indicated that the responses to carbachol, KCl and electrical field stimulation were highly repeatable between tissue strips from the same animal, and between animals. This finding is critical to the proposed canine studies as it means that any variation seen is likely to be due to biological variation and not to equipment or personnel. Prior to the use of the chosen experimental approach to study differences in detrusor function as a consequence of neutering in the canine, a series of studies were necessary to determine the exact experimental conditions required to optimise the conditions for use with this tissue. Due to the lack of reported data on canine detrusor muscle function, a length tension study on isolated strips of canine urinary bladder smooth muscle was conduced to determine the optimum resting tension for this tissue. These studies were also considered important as the canine bladder is significantly larger than that of the rat, and its function is different as domestic canines are ‘housetrained’ and thus require a significant storage function in addition to contractile micturition and thus may require different study conditions than that of the rat. Optimisation of the resting tension also allows more accurate assessment of the contractility of the bladder as the actin and myosin filaments are optimally aligned; if the

tension is too low then the filaments overlap, and if the tension is too high the filaments are not able to interact with each other properly, thus the contractile force of the stimulated muscle is decreased.

As all of the canine tissue was donated, with full owner consent, it was recognised that there would not be a constant or regular supply of tissue, and that tissue would often be donated late in the evening or at weekends. Given the larger size of the canine urinary bladder and to be able to utilise the tissue obtained fully, a further aim of the initial characterisation studies was to investigate the effect of storage of the tissue to determine if experiments could be run up to 3 days post harvesting, instead of on the day of harvesting as in the rat. It was hypothesised that changes would occur in the muscle tissue upon storage and that the contractility of muscle would diminish over time due to the build-up of waste products of metabolism and the depletion of oxygen and glucose within the storage solution by the tissue, all leading to degradation of the muscle tissue. The time course for these changes has not been previously reported, therefore tissue was used from the same animal on days 1, 2 and 3 post harvesting, with day of harvesting considered as day 0, and statistical analysis of the results performed to determine if tissue could reliably be used for more than one day.

3.2 Materials and Methods

3.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissue from a total of 22 canines was included in the study, with a mean age of 6 years (range 1-14 years) and a mean weight of 24.4kg (range 8-45kg). The majority of canines included in this study were cross bred, with no pedigree breeds appearing more than once. In all cases tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbatone), with full, informed, owner consent. In all cases, euthanasia occurred for reasons other than scientific investigation. The majority of animals were destroyed for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system.

3.2.2 Preparation of Tissue

A full, detailed history of each animal was taken and a gross post-mortem of the entire urinary tract performed, before the urinary bladder was harvested, sectioned across the level of the ureters and the dome of the bladder placed in Krebs solution (NaCl 118mM, KCl 4.8mM, $CaCl_2$ 2.5mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $NaHCO_3$ 24mM, glucose 11mM) and stored at 4°C. Any animal with a history of, or gross pathological signs of, urinary tract disease, other than acquired urinary incontinence, were excluded from the study.

The domes of the bladders were cleared of any fat and the lining urothelium removed. Bundles of smooth muscle fibres could then be identified and up to 28 strips of smooth muscle (2mm x 10mm) were produced from the resultant detrusor muscle sheet. The detrusor muscle strips were mounted in 15ml tissue baths (Scotia Glassware Ltd, Grangemouth, UK), bathed at 37°C in Krebs solution and continually aerated with 95% O_2 / 5% CO_2. Muscular tension was measured with BIOPAC TSD125C isometric force microtransducers (BIOPAC Systems Inc, California) and displayed on a computer screen using the BIOPAC data acquisition software (AcqKnowledge 3.8). To determine optimal resting tension, detrusor muscle strips from each animal were mounted under 2, 4, 6 or 8g resting tension. Tissue strips were washed (repeatedly) and allowed to equilibrate for 60

minutes, during which time the resting tension was adjusted on a per strip basis to achieve the intended passive muscle tension.

Initially, to assess tissue viability, muscle strips were exposed to a KCl challenge (40mM) and tissue strips that did not respond were discarded and replaced; however, it was found that response, or more frequently lack of response, to this initial dose of KCl did not accurately correlate to later contractile responses to carbachol and therefore this practice was stopped and no further test doses used. Tissues were then subjected to an assessment of carbachol induced contraction (3.2.3) followed by KCl induced contraction (3.2.4).

To study the effects of storage on the viability of tissue the bladder domes were kept in Krebs solution at 4°C for up to 3 days post harvesting. As it was hypothesised that muscle strip contractility would decrease over time due in part to utilisation of the components of the Krebs solution, and due to build up of waste products from metabolism the Krebs storage solution was refreshed daily. On days 1, 2 and 3 bundles of smooth muscle were dissected out as described above and subjected to an assessment of carbachol-, followed by KCl- induced contraction as described below.

3.2.3 Assessment of Carbachol-Induced Contraction

Once tension in strips was stable at the intended levels a carbachol concentration response protocol was performed. Carbachol (as per chapter 2) was added to the baths in a stepwise manner to give cumulative bath concentrations of 1nm – 30µM (log -9M to -4.5M), increasing in half log increments after the response to the previous concentration had reached a plateau.

3.2.4 Assessment of KCl-Induced Contraction

Following completion of the cumulative response protocol to carbachol, the detrusor muscle strips were washed repeatedly and allowed to equilibrate for 1 hour, during which time the resting tension was adjusted as necessary every 10 minutes to achieve the intended resting muscle tension for each strip. Once the strips of urinary bladder were stable at their designated resting tension, KCl was added to the baths in a stepwise manner to give final cumulative bath concentrations of 10mM – 80mM. This was performed using 10mM

increments with each further concentration applied after the response to the previous concentration had reached a plateau.

3.2.5 Data Analysis

Results were normalised relative to tissue weight and results presented as g/mg of wet tissue. Data are expressed as mean ± s.e.mean (n = number of canines).

Results from a minimum of 2 strips of bladder tissue per animal were analysed and the mean of these strips used in further calculations where applicable. Data were graphed and statistical analysis was performed using either GraphPad Prism® v.5, or GraphPad InStat 3 software. Comparisons between maximal tensions produced and age and weight of the animal were made using analysis of variance (ANOVA). Comparisons between day 1, 2 and 3 data were made using repeated analysis of variance (ANOVA) with Bonferroni post-test. A probability (P) less than or equal to 0.05 was considered significant.

3.3 Results

Carbachol induced concentration dependent contraction of the isolated strips of detrusor muscle from all animals (Fig. 3-1). Contraction was measurable at 10nM and reached a maximum at 3µM in all groups after which there was dose dependant relaxation seen in a number of strips (Fig. 3-1). The highest maximal contraction and lowest $LogEC_{50}$ value were seen in the strips placed at 4g resting tension (Table 3-1). Although the results for 4g were not significantly greater compared to the other tensions studied, it was noted that more strips at 6g and 8g resting tension either broke or failed to respond during the protocol (17% and 12% of strips respectively) compared to those at 2g and 4g resting tension (4% and 5% respectively). Analysis of variance showed no affect of age or weight of animals on the results obtained.

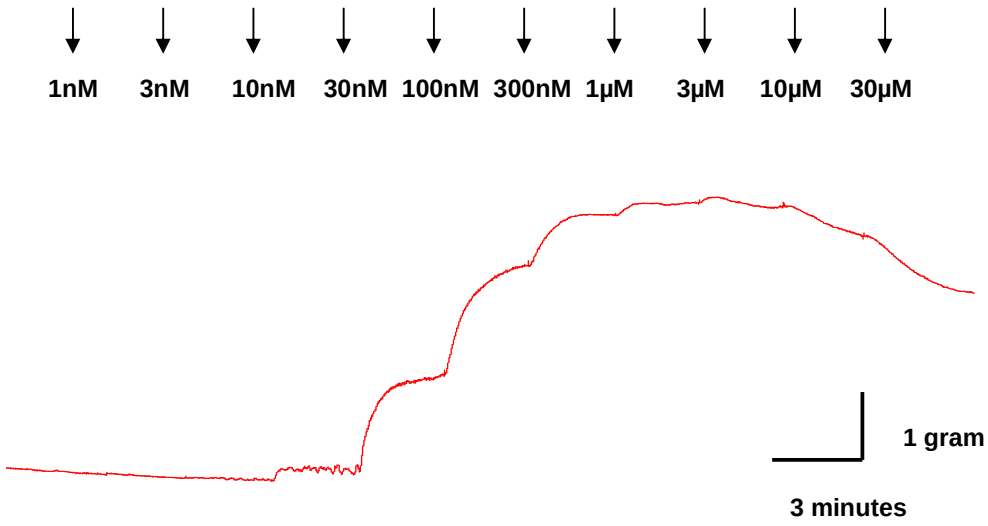

Figure 3-1. A representative section of trace showing the contractile response to carbachol in an isolated strip of canine detrusor muscle. Arrows denote addition of carbachol.

	Resting Tension			
	2g	4g	6g	8g
Carbachol – Emax	0.73 ± 0.12	0.94 ± 0.14	0.85 ± 0.13	0.83 ± 0.12
Carbachol – $LogEC_{50}$	-6.77 ± 0.14	-6.88 ± 0.14	-6.78 ± 0.13	-6.75 ± 0.12
KCl – Emax	0.16 ± 0.02	0.30 ± 0.06	0.25 ±0.06	0.28 ± 0.06

Table 3-1. Maximum (Emax) and Log EC_{50} values for Carbachol and KCl in isolated strips of canine detrusor muscle at different resting tensions. Data are expressed as g/mg of wet tissue with results given as mean ± s.e.mean. In all groups n = 22 animals.

KCl induced a dose dependent contraction of all isolated urinary bladder strips (Fig. 3-2). The mean maximal contraction produced by the strips of detrusor muscle in response to KCl was lower than that produced in response to carbachol (31%). Contraction was measurable at 10mM and reached a maximum at 30mM, in all groups (Fig 3-3b), after which there was dose dependant relaxation. The highest maximal contraction was seen in detrusor strips placed at 4g resting tension (Table 3-1). As with carbachol the number of detrusor strips that either broke or did not respond to the protocol was higher in the strips at 6g (11%) and 8g (24%), compared to 4g (7%) and 2g (10%) resting tension. Analysis of variance again showed no affect of age or weight of animal on the results gained.

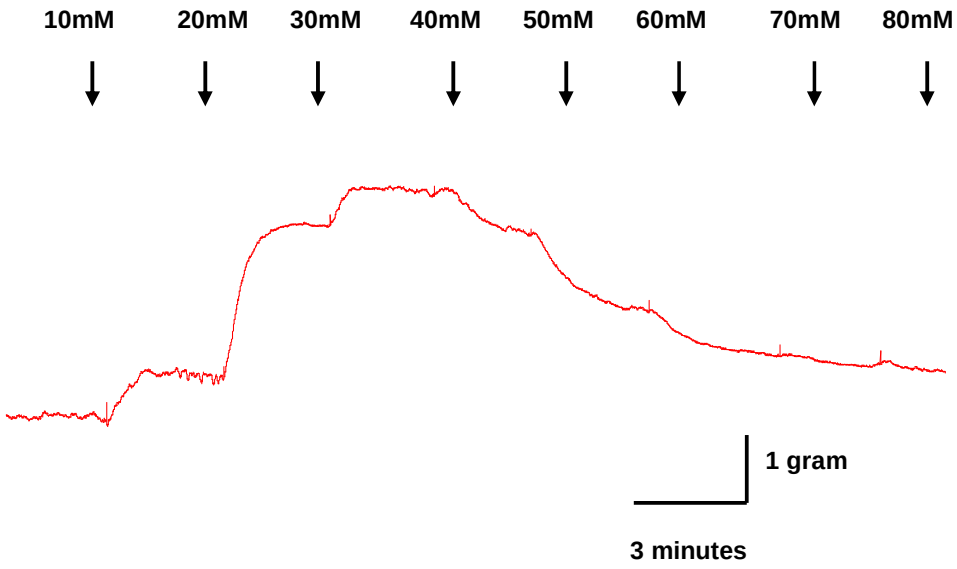

Figure 3-2 A representative section of trace showing the contractile response to KCl in isolated strips of canine detrusor muscle. Arrows denote addition of KCl.

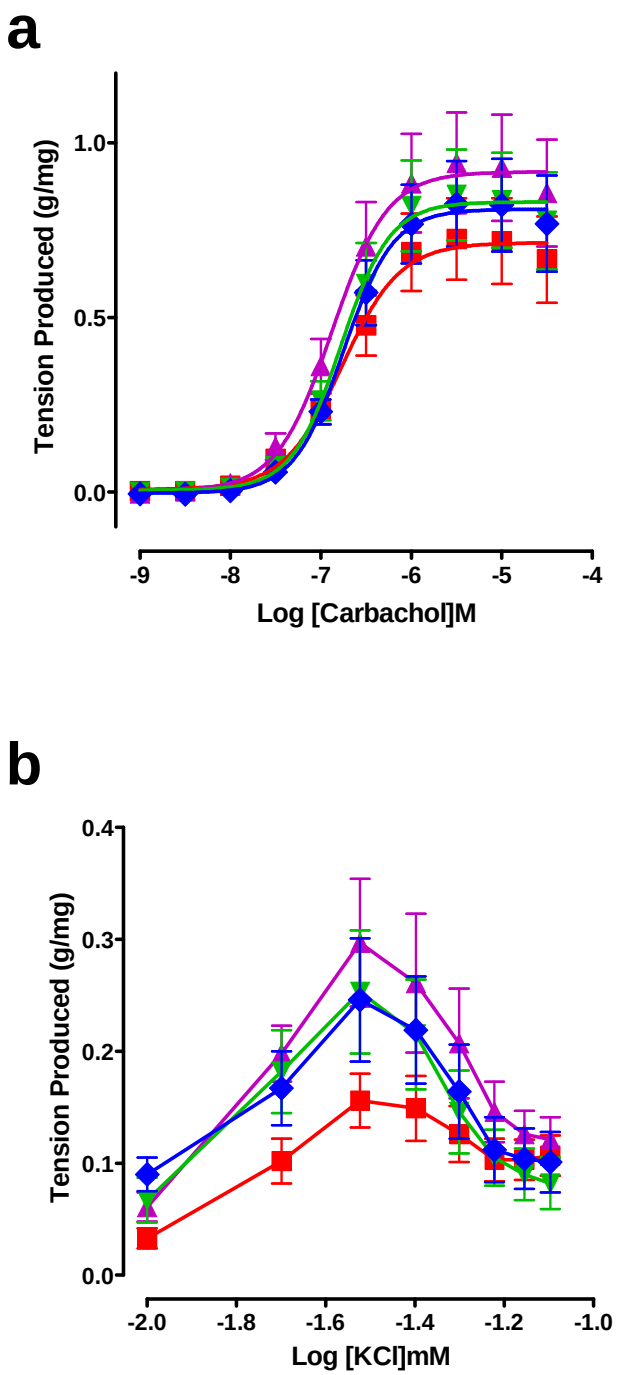

Figure 3-3 Cumulative concentration response curves to a, carbachol and b, KCl in isolated strips of canine detrusor muscle. Each point is the mean ± s.e.mean of observations from 22 animals. ■ 2g resting tension, ▲ 4g resting tension, ▼ 6g resting tension, ♦ 8g resting tension.

As strips placed under 4g resting tension gave the best results in the length tension studies, only these results were used to compare the affect of storage on contractile responses. Carbachol produced concentration dependant contraction of strips on all days studied (Fig. 3-4a). The maximal responses to carbachol on days 1 and 2 were similar (1.063 ± 0.289g/mg and 1.021 ± 0.344g/mg respectively), however, the maximal responses seen on day 3 (0.365 ± 0.107g/mg) were significantly lower than those seen on days 1 and 2 ($P < 0.001$). There was no significant difference between the $LogEC_{50}$ values on any of the days studied.

As for the length tension study, KCl produced concentration dependant contraction of detrusor muscle strips on all days studied (Fig. 3-4b). The pattern in maximal contraction to KCl was comparable to that of carbachol, with the maximal contraction on days 1 and 2 being similar (0.421 ± 0.040g/mg and 0.429 ± 0.065g/mg respectively) but being significantly decreased ($P < 0.001$), on day 3 (0.095 ± 0.015g/mg) compared to days 1 and 2.

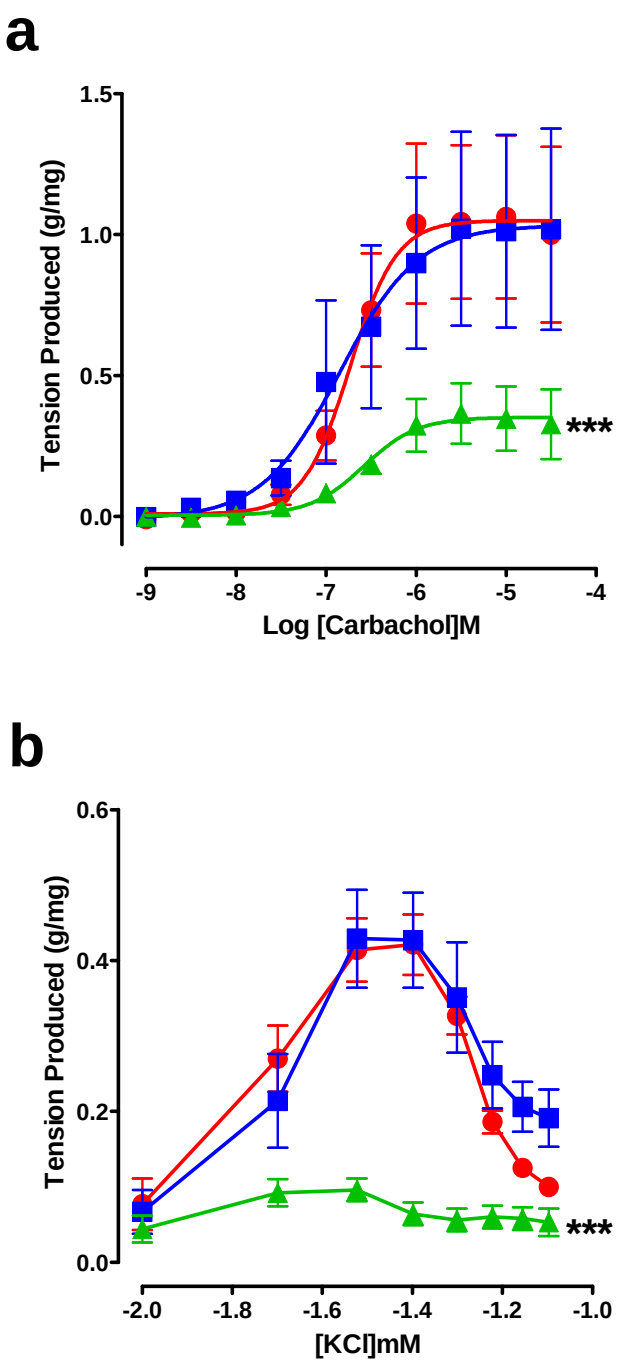

Figure 3-4. Cumulative concentration curves to a, carbachol and b, KCl in isolated strips of canine detrusor muscle at 1 day ●, 2 days ■, and 3 days ▲ post harvesting of tissue. Each point is the mean ± s.e.mean of observations from 5 animals. *** P<0.001 compared to other groups.

3.4 Discussion and Conclusion

Canine detrusor muscle responded to carbachol in a manner similar to that of other species studied (Fetscher *et al.*, 2002; Hawthorn *et al.*, 2000; Schneider *et al.*, 2004), in that there was concentration dependant contraction of the smooth muscle strips. By plotting the concentration dependant contractile responses to carbachol on a logarithmic scale a classical sigmoid curve was produced which allowed comparison between responses in terms of maximal contractile response seen and $LogEC_{50}$ values, which gave an indication of the sensitivity of the tissue to the agonist. When comparing the responses of the canine detrusor muscle to carbachol under differing resting tensions, it was found that although the maximal responses seen differed for the various resting tensions, the $LogEC_{50}$ values were comparable between groups. This similarity in $LogEC_{50}$ values is to be expected as all strips were being tested against the same muscarinic agonist, and would therefore be expected to show similar sensitivities as the binding properties and receptor responses should be equal in all strips.

Carbachol is thought to act mainly at the M_3 receptors in the bladder, which are the receptors deemed responsible for the majority of the contractile action of the bladder during micturition (Chess-Williams, 2002); however, it is possible that at higher concentrations carbachol may act on the M_2 and nicotinic receptors causing a change of state of the cation channels on the muscle cell membrane (Andersson *et al.*, 2004a; Rang *et al.*, 2007), and potentially altering both K^+ and Ca^{2+} conductance across the cell membrane. The change in state of K^+ ion channels, in particular, may explain why at higher concentrations (10µM and 30µM), carbachol was seen to invoke concentration dependant relaxation of the urinary bladder strips in this present study (Oh *et al.*, 2003).

Response of the canine detrusor muscle to KCl in this system was also as for other species studied (Chaiyaprasithi *et al.*, 2003; Hawthorn *et al.*, 2000). KCl is a metal halide that disassociates in solution to form K^+ (the major cation of cells) and Cl^- ions, both of which are essential to many body systems and functions including conductance of nerve impulses, acid-base balance and carbohydrate metabolism. KCl is not specific for any receptor but is thought to act directly upon the cell membrane (Foster *et al.*, 1983). When added to the extracellular environment KCl, at low concentrations, causes depolarisation of the cell membrane thereby leading to formation of action potentials and contraction of smooth muscle cells. At higher concentrations (>40mM) it is possible that alterations in relative concentrations of K^+, Ca^{2+}, Na^+ and Mg^{2+} ions may cause the hyperpolarisation of

the cell membrane and activation of various ion channels thereby inhibiting further contraction (Foster *et al.,* 1983; Kravtsov *et al.*, 1995), and eventually leading to relaxation.

When comparing the responses of the canine detrusor muscle to KCl, under differing resting tensions, it was found that although the maximal responses seen differed between the groups, the overall shape of the graphs was similar, with maximal response seen at 30mM KCl, followed by gradual concentration dependant relaxation at higher concentrations. This suggests that the overall mechanisms responsible for contraction and relaxation of the detrusor muscle strips are similar for all resting tensions studied.

The difference in maximal response to both carbachol and KCl seen between differing resting tensions is due to the effects of muscle length on the ability of the actin and myosin filaments within the thick and thin filaments to interact. The interactions between these filaments cause shortening of the muscle fibre when the muscle is stimulated (Andersson *et al.,* 2004a), which is evident as contractile tension in our system. There is an optimum arrangement of these thick and thin filaments, which, when stimulated, produces maximal contraction of the muscle. The resting tension at which this optimum alignment of thick and thin filaments within the smooth muscle will occur may vary between species and between different muscular organs as the *in vivo* passive tension normally placed upon the muscle will vary depending on species and organ. If the resting tension is too low, the thick and thin filaments overlap to such a degree that further shortening of the muscle fibre to produce contraction is inhibited (Andersson *et al.,* 2004a). This is seen in this study by the fact that the group under 2g resting tension had the lowest maximum response and at this resting tension a number of strips failed to response to carbachol or KCl. If the resting tension is too high, the thick and thin filaments are too far apart and therefore cannot interact properly to cause shortening of muscle fibres and contraction of the muscle when stimulated. This was evident in the 6g and 8g groups in this study, which showed a lower maximal response than the 4g group. The 6g and 8g groups also showed a greater number of strips breaking during contraction than the 2g and 4g groups. For canine detrusor muscle it would therefore appear that 4g resting tension is the optimal tension for further *in vitro* studies, as this was the tension that gave the maximal response to both carbachol and KCl, with the lowest number of strips breaking or failing to respond to treatment. This is in contrast to the 1-2g standard resting tension used in rat, rabbit and guinea pig (Kories *et al.*, 2003; Longhurst *et al.*, 2000; Longhurst *et al.*, 2001), but within the range of 1-4g resting tension suggested for use in studying human detrusor function *in vitro* (Yoshida *et al.,* 2006). This similarity to human tissue is unsurprising as the canine urinary bladder

shows greater similarity in terms of size and function to that of the human compared to that of rats, rabbits and guinea-pigs.

As canine tissue was donated supply was unpredictable, therefore, to optimise the system, a further study was conducted to determine the length of time, post tissue harvesting, that canine detrusor muscle would reliably and reproducibly respond in the above protocols. The results obtained demonstrated that tissue is viable for up to three days post harvesting but that contractile responses to stimulation significantly decrease on the third day. These results also demonstrated that responses to stimulation to both carbachol and KCl were similar on the first and second day post harvesting. From this it has been concluded that tissue may be stored and strips dissected out daily for use in protocols for two days after it has been harvested without affecting the responsiveness of the tissue. This allowed maximal use of all tissue donated for use in further studies.

As in the rat study in chapter 2, within-animal variation in the canine study was low; however, in contrast to the rat study, inter-animal variation was seen to be much higher in the canine. As the techniques and equipment used were validated by the study in the rat (chapter 2) the variation in response between canines is not due to equipment or personnel but due to the animals themselves. The rat population was consistent in terms of weight, age and strain of animal used, however, due to the constraints of the study this was not the case in the canine. Analysis of variance showed that there were no significant effects of age or weight of the animal on the maximal contraction of the detrusor muscle strips to either carbachol or KCl, therefore the variation between animals cannot be due to these factors. Although the breed of the animal may have a role to play in the variation seen, the majority of canines in the study were cross bred, thereby keeping specific breed variation to a minimum. Previous studies in the rat have suggested that there may be functional changes in the bladder due to gender or ovariectomy (Fleischmann *et al.*, 2002; Longhurst *et al.*, 2000) therefore it was hypothesised that the variation seen between animals could be due to gender and neutering as male and female, entire and neutered animals were included in the study.

In conclusion the study showed that 4g initial resting tension is the optimum resting tension for use in tissue bath studies that use the canine detrusor muscle, and that strips of this smooth muscle can be used for two days post harvesting without affecting the results gained. Furthermore, high inter-animal variation in the responses of isolated strips of detrusor muscle to carbachol and KCl stimulation seen in this study suggest significant

differences in tissue responsiveness and sensitivity exist between canines, possibly due to gender or gonadal differences.

4 Effects of Neutering on the *in vitro* Responses of Canine Detrusor Muscle Strips to KCl, Carbachol and Electrical Stimulation

4.1 Introduction

Urinary incontinence is defined as the involuntary loss of urine (Abrams *et al.*, 2002), and is a severely debilitating condition that has significant welfare implications for affected patients. In the canine, the most commonly seen form of urinary incontinence is acquired urinary incontinence also known as post neutering incontinence, as it is seen most frequently in bitches after they have been neutered. Indeed, acquired urinary incontinence is seen in up to 20% of all neutered bitches (Arnold *et al.*, 1989) whilst less than 1% of entire bitches and males are affected (Holt *et al.*, 1993). Acquired urinary incontinence has been directly linked to being neutered (Thrusfield, 1985), although mechanical damage of the lower urinary tract, during surgery, is not implicated in the aetiology (Gregory, 1994). A number of other hypotheses for the causes of post-neutering acquired urinary incontinence have also been put forward, including vascular, neurological and hormonal changes, the later of which is currently the subject of research. Acquired urinary incontinence in the bitch is thought to have a similar aetiology to that of post menopausal incontinence in women, although the exact pathophysiology of the condition in these two groups is not well enough understood to allow determination of commonality of cause.

It has been shown in women that although the resting tone of the urethra may be involved in post menopausal urinary incontinence, the urinary bladder also has a significant role to play. Studies have shown that there is impaired contractility of the bladder in women suffering from post menopausal urinary incontinence (Elbadawi *et al.*, 1993a) and that this is frequently found in conjunction with idiopathic detrusor instability (Elbadawi *et al.*, 1993b; Resnick *et al.*, 1987). In patients suffering from impaired bladder contractility a change in responsiveness and contractility of the detrusor muscle to muscarinic stimulation has been demonstrated *in vitro* (Mills *et al.*, 2000) and these finding have been replicated in neutered female rats (Fleischmann *et al.*, 2002; Zhu *et al.*, 2001). These aspects of bladder function in the canine have never been reported.

Interestingly, during the validation studies reported in chapters 2 and 3 it was noted that there was a greater degree of variation in responsiveness of the detrusor muscle strips to

both carbachol and KCl stimulation in canines than in the rat. Although there will be a degree of biological variation in response due to the diversity of the canine population studied, I hypothesise that a significant proportion of the variation seen in the canine study may be due to gonadal and / or gender differences in the study population. As discussed above, *in vitro* studies have shown that gonadal status influences bladder contractility in both the human and the rat and that this change in contractility has been hypothesised to be linked to urinary incontinence in postmenopausal women (Mills *et al.*, 2000). A similar change in contractility of the detrusor in the canine may, therefore, be involved in the development of acquired urinary incontinence in neutered bitches. It is of note that in the majority of the studies so far reported looking at the role of gonadal status on the function of the detrusor muscle in all species; the emphasis has been on the female, with only a few studies considering the changes that may occur in the bladder of male subjects after gonadectomy. In particular, there are no studies looking at the contractility of the urinary bladder after gonadectomy in the male to see if similar functional changes occur as have been reported in the female.

It is known that the muscarinic pathway is the primary pathway responsible for bladder contraction in the mammal (Chess-Williams, 2002). Studies looking at the contractility of the detrusor muscle *in vitro* therefore seek to stimulate the muscarinic pathway either via muscarinic agonists, such as carbachol, or via neurogenic field stimulation. It is also known, however, that part of the neuronally induced bladder contraction is resistant to atropine (Andersson *et al.*, 2004a) and is therefore out-with cholinergic control. This atropine resistant component of contraction is termed the non-adrenergic, non-cholinergic response and its proportion relative to the total contraction of the detrusor varies with species and disease state (Andersson *et al.*, 2004a). In humans this non-adrenergic, non-cholinergic mediated response can contribute up to 65% of the total contraction in patients suffering from urinary incontinence due to detrusor overactivity, over twice that of control patients (Andersson *et al.*, 2004b). It is thought that the non-adrenergic, non-cholinergic response may be mediated by ATP, at least in the human (Andersson *et al.*, 2004a; Palea *et al.*, 1993). There have been no reported studies looking at the role of the non-adrenergic, non-cholinergic system in the canine detrusor however.

In this study the hypothesis that gonadal status and / or gender will alter the *in vitro* contractility and responsiveness of isolated strips of canine detrusor muscle to non-specific, muscarinic and electrical field stimulation was tested. The further hypothesis that the non-adrenergic, non-cholinergic system has a role to play in detrusor contractility in the canine was also tested. Based on data from the human and the rat it was expected that the

responsiveness and contractility of the detrusor would be lowest in neutered animals and that the role of the non-adrenergic, non-cholinergic system would be greatest in gonadectomised animals.

4.2 Materials and Methods

4.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissue from a total of 63 canines was included in the study, although not all animals were included in each protocol. The study population had a mean age of 5.9 ± 0.5 years (range 1-14 years) and a mean weight of 22.9 ± 1.3kg (range 8-48kg). The canines were split into five groups depending on gender, gonadal status and incidence of acquired urinary incontinence: entire and neutered males, entire and neutered females, plus neutered females known to be suffering from acquired urinary incontinence (determined by history and signs of urine scalding around perineum). The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases, tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbatone), with full informed owner consent, for reasons other than scientific investigation. The majority of animals had been euthanized for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system.

4.2.2 Preparation of Tissue

A full, detailed history of each animal was taken and a gross post-mortem of the entire urinary tract performed before the urinary bladder was harvested, sectioned across the level of the ureters, the dome of the bladder placed in Krebs solution (NaCl 118mM, KCl 4.8mM, $CaCl_2$ 2.5mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $NaHCO_3$ 24mM, glucose 11mM) and stored at 4°C. Any animal with a history of, or gross pathological signs of, urinary tract disease, other than acquired urinary incontinence, was excluded from the study.

For all tissue bath protocols bladder tissue was used within 2 days of euthanasia of the animal (see chapter 3).

Preparation of detrusor strips was as for chapter 3 with the addition of strips of detrusor muscle being mounted in Ag-AgCl ring electrodes (manufactured by Mr I. Gibson, Glasgow University Veterinary School) for electrical stimulation protocols (4.2.5 and 4.3.6), as well as on fixed hooks for KCl and carbachol concentration response protocols (4.2.3 and 4.2.4 respectively).

4.2.3 Assessment of KCl-Induced Contraction

This was as described in chapter 3, with the exception that all strips of detrusor muscle were placed under 4g resting tension as this was deemed to be optimal (chapter 3).

4.2.4 Basic Carbachol Concentration Response Protocol

This was as described in chapter 3, with the exception that all strips of detrusor muscle were placed under 4g resting tension as this was deemed to be optimal (chapter 3).

4.2.5 Basic Neurogenic Electrical Field Stimulation Protocol

Strips of detrusor muscle were dissected as previously described, mounted on a fixed hook and passed through an Ag-AgCl ring electrode. Strips were washed repeatedly with oxygenated Krebs as in the Carbachol and KCl protocols and tensioned until stable at 4g resting tension. Once stable, the supramaximal voltage (at 20Hz) was determined for each strip by application of electrical stimulation (100 pulses) with increasing voltages until the maximal contractile response was seen; the voltage required to produce approximately 70% of this response was considered the supramaximal voltage of that strip. A train of three stimulations at this voltage was then conducted to test that the response seen at that voltage was stable.

Strips were then washed, re-tensioned to 4g resting tension and allowed to equilibrate for 30 minutes, during which time the strips were re-tensioned to 4g every 10 minutes. Once stable, electrical field stimulation (0.5-100 Hz, 100 pulses) was delivered from a Digitimer Ltd MultiStim System-D330 stimulator at a pulse width of 0.5ms and at supramaximal voltage. Stimulations were applied at five minute intervals to allow the re-establishment of normal resting tone between stimulations. Frequency dependant contractions were observed. To confirm that the observed responses to electrical field stimulation were neurogenic, at the end of the first three experiments for each group of animals the tissue strips were incubated with the neurotoxin Tetrodotoxin (1μM) (Sigma, UK) and stimulated at 20Hz every 5 minutes for 3 stimulations.

4.2.6 Non-Adrenergic, Non-Cholinergic Neurogenic Electrical Field Stimulation Protocol

This was performed after the initial neurogenic field stimulation protocol described in 4.2.5 on strips that were not treated with Tetrodotoxin. In all cases a minimum of 4 strips from each animal were used.

After the initial frequency response protocol was carried out all strips were washed repeatedly, allowed to rest for 30 minutes and then tensioned until stable at 4g resting tension. A train of stimulations was then performed at 20 Hz and supramaximal voltage until 3 consecutive, stable responses were obtained. This train was continued in all strips, with 2 strips receiving no chemical additions to act as time controls. Atropine (Sigma, UK), a competitive antagonist at muscarinic receptors, was then added to the other baths to give a bath concentration of 1µM and the train of stimulation continued until 3 further stable contractions were obtained. α-, β- methylene ATP Atropine (Sigma, UK), an agent known to stimulate and then to desensitize P2-purinoceptors (Shinozuka *et al.*, 1990), was then added to these baths to give a final bath concentration of 10µM and the chain of stimulation continued until 3 further stable contractions were obtained.

4.2.7 Data Analysis

All data were normalised for wet weight of tissue and results expressed as mean ± s.e.mean tension (g/mg of wet tissue) (n = number of animals). Results for response to atropine and α-, β- methylene ATP are expressed as percentage of maximal, non-treated response.

A minimum of 2 strips of bladder tissue per animal were analysed and the mean results from these strips used in further calculations where applicable. Data were graphed and statistical analysis was performed using GraphPad Prism® v.5 software. In all cases the maximal tension was the mean maximal tension of all the strips from that animal. The $LogEC_{50}$ values were calculated by GraphPad Prism® v.5 software from the mean data for each animal. Comparisons between groups were made using analysis of variance (ANOVA) with Bonferroni post-test. A probability (P) less than or equal to 0.05 was considered significant. Correlations between responses to muscarinic and electric field stimulation and the age and weight of the animals, were conducted using Spearman's test with a significance threshold for P (two tailed) < 0.05.

4.3 Results

4.3.1 Response to KCl Stimulation

There was concentration dependent contraction in all urinary bladder smooth muscle strips, in response to KCl. Contraction was measurable at 10mM and reached a maximum at 40mM, with the exception of the neutered females suffering from acquired urinary incontinence where the maximum contraction was seen at 30mM (Fig. 4-1). Upon further addition of KCl, strips showed varying but progressive degrees of relaxation in all groups. Analysis of variance showed that there was no effect of gender on the contractile responses to KCl but that neutering was associated with a significantly decreased maximal response to KCl ($P<0.05$): entire females 0.34 ± 0.08g/mg (n = 6), neutered females 0.18 ± 0.04g/mg (n = 5), entire male 0.31 ± 0.06g/mg (n = 8), neutered males 0.16 ± 0.06g/mg (n = 7). There was no statistically significant interaction between the effects of gender and gonadal status on the KCl response of the tissue strips and no correlation between age or weight of the animal and maximal KCl values.

The group of neutered female animals suffering from acquired urinary incontinence (n = 3) had a lower maximal response (0.09 ± 0.005g/mg) than that of all of the other groups but this difference in response was not statistically significant compared to that of the continent neutered females (95% CI -0.031 to 0.133).

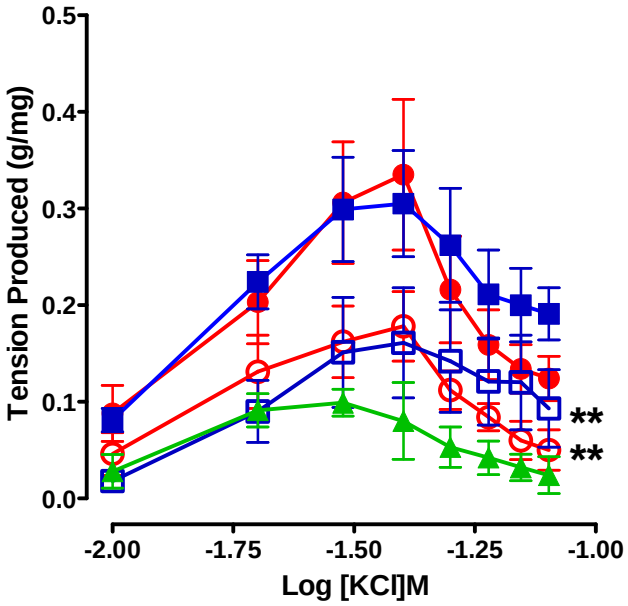

Figure 4-1. Cumulative concentration response curves to KCl in isolated canine urinary bladder smooth muscle strips. Each point is the mean ± s.e.mean of observations from n animals. ■ entire male, n = 8, □ neutered male, n = 7, ● entire female, n = 6, ○ neutered female, n = 5, ▲ neutered female known to be suffering from acquired urinary incontinence, n = 3. ** P<0.05 compared to animals of same gender.

4.3.2 Response to Carbachol Stimulation

In all groups, concentration dependant contractions were observed in response to carbachol (Fig. 4-2). Neutering, regardless of gender, was associated with a significant decrease in the maximum contractile response of the isolated strips of bladder smooth muscle to carbachol compared to entire animals ($p<0.05$). This change in maximum contractile response was accompanied by a significant decrease in sensitivity of the isolated strips of bladder smooth muscle to carbachol, as measured by the $LogEC_{50}$ values ($p<0.001$) (Table 4-1). The group of neutered females suffering from acquired urinary incontinence had lower maximal responses to carbachol and lower sensitivity, as measured by $LogEC_{50}$ values, than other groups, but neither maximal response or sensitivity were significantly lower than from the continent neutered females (95% CI -0.340 to 1.103 and -0.580 to 0.083 respectively). 2 way ANOVA indicated that there was no statistically significant effect of gender on the maximal responses to carbachol, and there was no interaction between gender and gonadal status on the response of the tissue to carbachol. In addition,

the statistical analysis indicated that there was no correlation between age or weight of the canines and the tissue responses to carbachol.

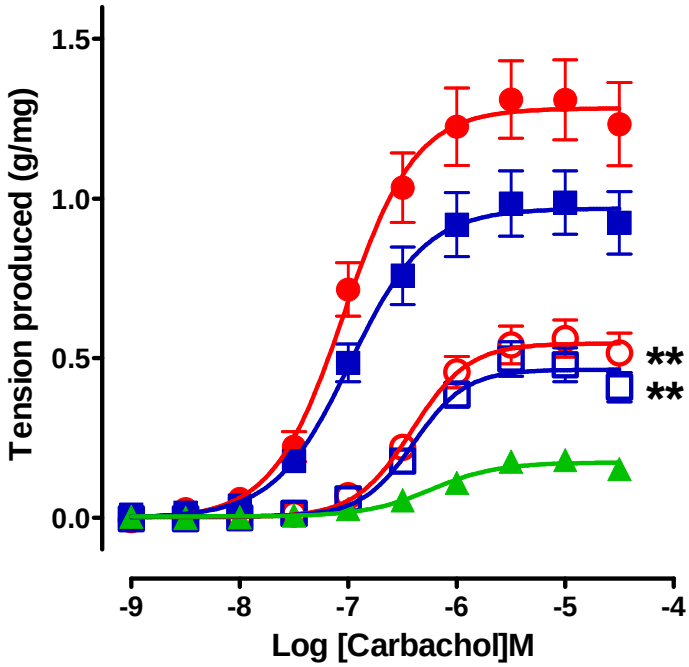

Figure 4-2. Cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips. Each point is the mean ± s.e.mean of observations from n animals. Where the s.e.mean cannot be seen they lie within the symbols area. ■ entire male, n = 15, □ neutered male, n = 11, ● entire female, n = 17, ○ neutered female, n = 8, ▲ neutered female known to be suffering from acquired urinary incontinence, n = 3. ** P<0.05 compared to animals of same gender.

	ME (n=15)	MN (n=11)	FE (n=17)	FN (n=8)	FN AUI (n=3)
Emax	1.04 ± 0.11	0.51 ± 0.06†	1.32 ± 0.14	0.56 ± 0.06††	0.18 ± 0.03
Log EC_{50}	-6.97 ± 0.04	-6.47 ± 0.05††	-7.02 ± 0.05	-6.51 ± 0.07††	-6.26 ± 0.06

Table 4-1. Maximum (Emax) and Log EC_{50} values for Carbachol in isolated strips of detrusor muscle from entire and neutered male and female canines (ME, MN, FE and FN respectively), and neutered female canines known to be suffering from acquired urinary incontinence (FN AUI). Data are expressed as g/mg of wet tissue with results given as mean ± s.e.mean where n = number of animals. † P<0.05, †† P<0.005 indicates different from entire of same gender.

4.3.3 Response to Neurogenic Field Stimulation

In all groups, frequency dependant contractions were observed in response to electrical field stimulation, with maximum tension observed at 20Hz. The effects of neutering on the response of isolated strips of bladder smooth muscle to neurogenic electrical field stimulation were similar to those of carbachol, with a significantly ($p<0.05$) decreased maximal contractile response seen in neutered compared to entire animals, regardless of gender (Fig. 4-3); entire males 0.50 ± 0.05g/mg (n = 15), neutered males 0.29 ± 0.03g/mg (n = 9), entire females 0.69 ± 0.10 (n = 10), neutered females 0.27 ± 0.03 (n = 7). Unfortunately, data was only obtained from two of the three neutered female animals suffering from acquired urinary incontinence, due to equipment failure. Maximal responses in these two animals were lower (Canine 22: 0.18g/mg, Canine 57: 0.26g/mg) than that seen in the neutered female group (95% CI is 0.264 to 0.362). As with the responses to carbachol, there was no statistically significant effect of gender on size of the maximal response, and there was no interaction between gender and gonadal status on the response of the tissue to neurogenic field stimulation. In addition the statistical analysis indicated that there was no correlation between age or weight of the canines and the tissue responses to neurogenic field stimulation.

A significant positive correlation ($P<0.001$) was noted between the maximal contractile responses of the strips of urinary bladder smooth muscle to carbachol and neurogenic field stimulation, for all of the animals for which both sets of data were available; 9 entire males, 8 neutered males, 9 entire females, 6 neutered females, 2 neutered females suffering from acquired urinary incontinence (Fig. 4-4).

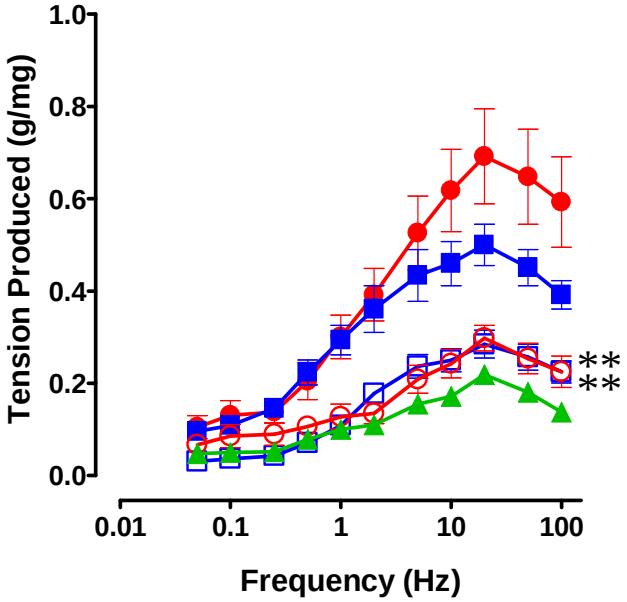

Figure 4-3. Frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips. Each point is the mean ± s.e.mean of observations from n animals. ■ entire male, n = 15, □ neutered male, n = 9, ● entire female, n = 10, ○ neutered female, n = 7, ▲ neutered female known to be suffering from acquired urinary incontinence, n = 2. ** P<0.05 compared to animals of same gender.

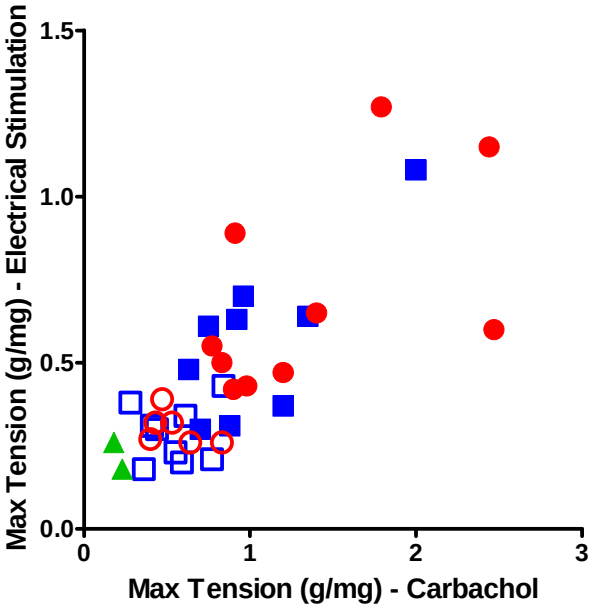

Figure 4-4. Graph showing the correlation between maximal tensions produced in response to carbachol and neurogenic field stimulation (correlation coefficient = 0.769). Each point is the combined data of one animal. ■ entire male, n = 9, □ neutered male, n = 9, ● entire female, n = 10, ○ neutered female, n = 6, ▲ neutered female known to be suffering from acquired urinary incontinence, n = 2.

4.3.4 The Non-Adrenergic, Non-Cholinergic System

In all animals there was minimal decrease in contractile response of the isolated strips of detrusor muscle to electrical field stimulation over time (<5% decrease).

In all groups atropine produced a reduction in the response to electrical field stimulation demonstrating that part of the responses seen were mediated via the muscarinic receptors. However, as the response was not abolished by atropine this also demonstrated that part of the responses seen were mediated by the non-adrenergic, non-cholinergic system (Fig. 4-5). The atropine resistant component of the contractile response to electrical field simulation was statistically similar in each group studied.

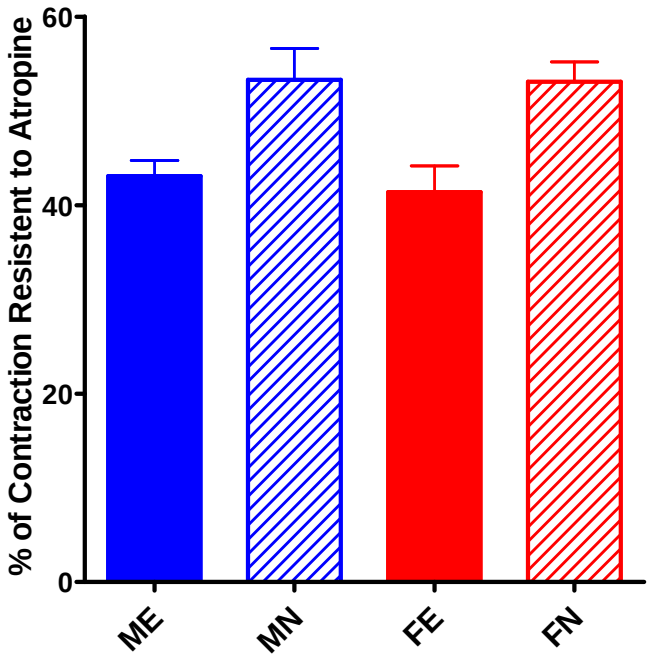

Figure 4-5. Graph showing the atropine resistant (non-adrenergic, non-cholinergic mediated) component of the electrical field stimulation induced contractile response of isolated strips of canine urinary bladder smooth muscle from entire male (ME, n = 5), neutered male (MN, n = 4), entire female (FE, n = 4) and neutered female canines (FN, n = 3).

In all groups α-, β-methylene ATP produced a similar reduction in the maximal contraction produced by the isolated strips of detrusor muscle via the non-adrenergic, non-cholinergic

system in response to electrical field stimulation (Fig. 4-6). Statistical analysis indicated that there was no difference in the size of the reduction between groups and that was no correlation between age or weight of the canines and the tissue responses to either atropine or α-, β-methylene ATP addition.

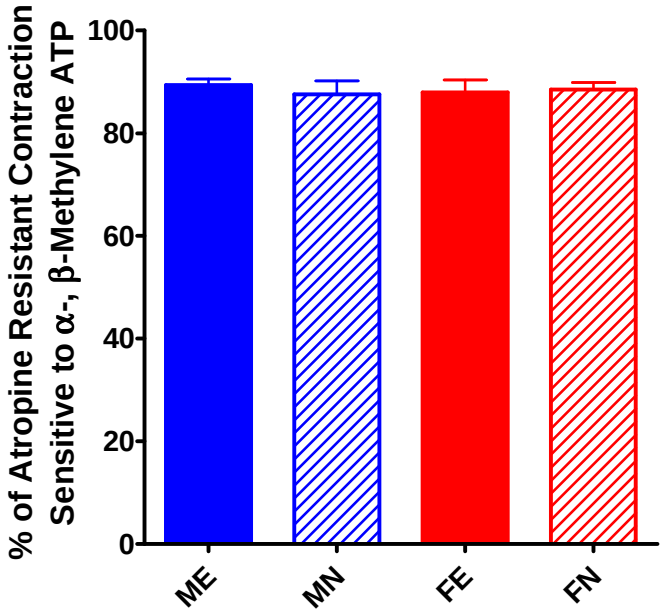

Figure 4-6. Graph showing the α-, β- ATP sensitive component of the non-adrenergic, non-cholinergic mediated contractile response of isolated strips of canine urinary bladder smooth muscle to electrical field stimulation. Groups are entire male (ME, n = 5), neutered male (MN, n = 4), entire female (FE, n = 4) and neutered female canines (FN, n = 3).

Over all carbachol, KCl, electrical field stimulation and non-adrenergic, non-cholinergic stimulation protocols it was noted that a small proportion of the isolated strips of detrusor muscle (minimum 8 strips per animal per protocol) failed to respond to stimulation. This was more noticeable in the neutered animals (average 25% of strips) compared to the entire animals (average 12.5% of strips), and clearly more pronounced in the animals suffering from acquired urinary incontinence (average 62.5% of strips) than in the other groups. There was no correlation between age or weight of the animal and the number of strips from that animal that responded.

4.4 Discussion

This study investigated the effects of neutering on the *in vitro* contractility of the smooth muscle of the canine urinary bladder in response to a variety of stimulatory systems, to determine whether functional changes occur following neutering that may be associated with acquired urinary incontinence in the bitch. This study further explored whether the non-adrenergic, non-cholinergic stimulation of detrusor activity had a role to play in electrical field stimulation induced contraction of the canine detrusor muscle *in vitro* and whether this differed between male and female, entire and neutered canines.

The results from this present study demonstrate that, regardless of gender, there is a marked decrease in the magnitude of the response of the smooth muscle of the urinary bladder wall to KCl, carbachol and neurogenic electrical field stimulation in neutered compared to gonad intact animals and that the responsiveness of the bladder wall is further decreased in animals known to be suffering from acquired urinary incontinence. In addition a decrease in sensitivity to carbachol, as measured by $LogEC_{50}$ values, was also observed in the detrusor muscle strips from neutered compared to entire animals. These changes could indicate a decrease in bladder muscle strength and contractility *in vivo* and thus could contribute to susceptibility to, and clinical signs of, acquired urinary incontinence.

The generalised decrease in responsiveness of the detrusor muscle from neutered canines observed in this study is similar to that described in human patients where impaired contractility of the detrusor muscle is recognised as a predisposing factor for urinary incontinence in post-menopausal women (Resnick *et al.,* 1987). In post-menopausal humans, impaired bladder contractility is characterised by slow contraction of the bladder with contractions being of decreased magnitude that lead to partial emptying of the bladder and consequent retention of urine (Resnick *et al.*, 1987), this is thought to predispose the person to developing urinary incontinence. It is thus hypothesized that the decrease in response of the canine detrusor muscle, shown in the present study may, similarly be a causative factor for acquired urinary incontinence in the neutered bitch.

KCl causes non-specific, receptor independent depolarisation of the cellular membrane leading to contraction of smooth muscle cells (see chapter 2). Thus, the decrease in responsiveness to KCl seen in the neutered animals in this study could indicate a generalised decrease in contractility of the bladder in these animals. As the response to KCl is further decreased in the animals known to be suffering from acquired urinary

incontinence it is possible that a decrease in bladder contractility could be at least partially responsible for the development of urinary incontinence in these animals. If this were to be correct, this decrease in the strength of bladder contraction at the time of urination could result in incomplete urination and an accumulation of urine within the bladder that may be voided inappropriately, for example when abdominal pressure increases or the bladder contracts inappropriately, as in idiopathic detrusor instability in women. Residual urine within the bladder may also lead to irritation of the bladder lining and wall and a propensity to develop cystitis, which is frequently seen in animals suffering from acquired urinary incontinence.

The effects of neutering on the response to KCl in female canines seen in this study are supported by work in the rat and rabbit which showed that long term ovariectomy can significantly reduce the responsiveness of the bladder to KCl *in vitro* (Lin *et al.*, 2006a; Longhurst *et al.*, 1992). Furthermore, work on the rabbit has shown that ovariectomy increases the RhoA / Rho-associated kinase (ROK-alpha) intracellular signalling pathway in the bladder (Lin *et al.*, 2006b) which is known to be involved in bladder contraction (Christ *et al.*, 2007). The increase in Rho/ROK-alpha following ovariectomy in the rabbit is thought to be a compensatory mechanism by which the bladder strives to maintain contractility and function (Lin *et al.*, 2006b), and the enhancement of this pathway may be responsible for the involuntary partial contraction of the bladder seen in patients diagnosed with idiopathic detrusor instability and associated urinary incontinence. Lin *et al.* (2006b) also demonstrated that ovariectomy results in a decrease in the smooth muscle to collagen ratio within the urinary bladder which may also contribute to the decrease in the overall contractile responses of these bladders (Lin *et al.*, 2006a).

Carbachol is a non specific muscarinic agonist that causes contraction of the bladder via activation of the muscarinic pathways which are thought to be the primary pathways responsible for bladder emptying (Chess-Williams, 2002) and the pathways stimulated by electric field stimulation (Creed et al., 1983; D'Agostino et al., 1989) (see chapter 2). This commonality of pathway regulation of contraction for both carbachol and electrical field stimulation is further supported by data which shows a positive correlation between maximal responses obtained to these stimulations in all animals studied. The differences in function within these pathways in entire and neutered canines observed in the current study could dramatically alter the bladder's ability to contract fully as a single functional unit and thus affect normal bladder function. This possibility is supported by the data from neutered female animals suffering from acquired urinary incontinence whose maximum

contractile response to carbachol fell below the 95% CI for the continent neutered female group.

Urinary incontinence is recognised as a multimodal condition in all species studied. In human patients urinary incontinence has been linked to decreased responsiveness of the detrusor muscle to muscarinic and electrical field stimulation *in vitro* that may be due to changes in the muscarinic receptor effector pathway, as described above, and idiopathic detrusor instability (Mills et al., 2000). Clinically idiopathic detrusor instability can lead to urge incontinence and inappropriate leakage of urine as bladder tone is unstable and thus partial contraction of the detrusor muscle, during the storage phase of micturition, can occur out-with the conscious and voluntary control of the patient (Mills et al., 2000; Resnick et al., 1989). Whilst idiopathic detrusor instability has not been studied functionally in the bitch, the results of this *in vitro* study demonstrate a decrease in the responsiveness of the bladder smooth muscle to neurogenic field stimulation in neutered canines, which is similar to that seen in human patients suffering from idiopathic detrusor instability, and thus is consistent with a role for detrusor instability in acquired urinary incontinence in the bitch.

As discussed previously acquired urinary incontinence in canines is gender specific (Arnold *et al.*, 1989; Aaron *et al.*, 1996). Interestingly this study demonstrated that changes in responsiveness to KCl, carbachol and electrical field stimulation occur in bladder smooth muscle in neutered canines of both genders. Whilst the functional changes observed in this study could support the development of acquired urinary incontinence in the neutered bitch, it is worthy of note that similar changes occurred in the bladder of neutered male canines, which do not typically become incontinent. This sex difference in the propensity to develop acquired urinary incontinence despite similar changes in detrusor contractility could reflect an interaction between post neutering effects on smooth muscle function and known anatomical differences in the urethra of male and female canines, that make the development of acquired urinary incontinence less likely in the male. Namely, the increased urethral length and passage of the urethra through the prostate and penile structures in the male is thought to provide a greater and more consistent urethral closure pressure than in the female (Aaron *et al.*, 1996), and this could counter the effects of neutering on the detrusor muscle and help explain why urinary incontinence is less prevalent in neutered male canines.

Although the primary pathway responsible for bladder contraction is known to be the muscarinic pathway, mediated via cholinergic neurotransmitters, there is evidence that a

proportion of contraction may be mediated via other mechanisms such as the adrenergic and non-adrenergic, non-cholinergic mechanisms. It is known that in most mammalian species part of the neuronally induced bladder contraction is resistant to atropine (Andersson, 1993). This atropine resistant component of contraction is termed the non-adrenergic, non-cholinergic mediated response and it's contribution to the total contraction has been reported to vary with both species and the frequency of stimulation used in *in vitro* studies. The results of the present study suggest that this mechanism has a role to play in mediating contraction in the canine urinary bladder and that it may mediate up to 50% of the electrically stimulated response. It has been reported that the non-adrenergic, non-cholinergic system may be responsible for up to 75% of the contractile response in isolated strips of detrusor muscle from rats (Andersson *et al.*, 2004a) but in rabbits and pigs this figure falls to 60 and 25% respectively (Brading *et al.*, 1991). In the human bladder the role of the non-adrenergic, non-cholinergic mechanism is still disputed (Andersson *et al.*, 2004a), however, it has been reported that the non-adrenergic, non-cholinergic mechanism is responsible for between 0 and 50% of the nerve mediated contraction of the urinary bladder in the human (Cowan *et al.*, 1983; Sibley, 1984).

This present study also demonstrated that although the maximal contraction to electrical field stimulation varied with gonadal status, the proportion of the response mediated via the non-adrenergic, non-cholinergic system was not significantly different in all groups studied, although a trend was seen for the non-adrenergic, non-cholinergic component of electrical field stimulated contraction to be less in entire animals. This trend agrees with the original hypothesis that stated that gonadectomised animals would have a significantly higher proportion of the contractile response mediated by the non-adrenergic, non-cholinergic system, although further study including larger numbers of animals should be conducted before further analysis and conjecture. The original hypothesis was based on a number of reported studies looking at the role of the non-adrenergic, non-cholinergic mechanism in bladder contraction in certain disease states in humans, namely bladder outflow obstruction and detrusor overactivity that resulted in urinary incontinence. These studies have reported a variety of results but agreed that the atropine resistant component of electrically induced contraction was much greater (25 – 65%) in association with bladder outlet obstruction and detrusor overactivity than in controls (Andersson *et al.*, 2004b).

The functional neurotransmitter(s) responsible for the non-adrenergic, non-cholinergic mechanism have not yet been fully identified, however a number of compounds have been proposed. These include Adenosine-5'-triphosphate (ATP), nitric oxide, various

neuropeptides and prostanoids. Of these, ATP has been the most extensively studied and demonstrates the most compelling evidence for involvement in the non-adrenergic, non-cholinergic mechanism of bladder contraction (Andersson *et al.*, 2004a). The present study demonstrated that the majority of the non-adrenergic, non-cholinergic mediated response in the canine, regardless of gender and gonadal status, is mediated by ATP. This result agrees with a number of studies in humans and guinea-pigs which have demonstrated that the atropine resistant portion of contraction can be blocked by α, β-methylene ATP (Andersson *et al.*, 2004a; O'Reilly *et al.*, 2002; Palea *et al.*, 1993). It is known that ATP works via stimulation of P2X receptors (Andersson *et al.*, 2004a), and it has been shown that the P2X receptor subtypes are present in the human bladder (Hardy *et al.*, 2000; O'Reilly *et al.*, 2002). Further studies have shown that other animals, such as the rat, mouse, rabbit and cat have multiple purinergic excitatory receptors present within the bladder as well (Andersson, 1993). It is hypothesised that ATP mediates detrusor contraction via activation of a ligand-gated cation channel (the P2X receptor) that promotes the influx of extracellular Ca^{2+} (Andersson *et al.*, 2004a). This all suggests that ATP may contribute to excitatory neurotransmission within the bladder, and that the purinergic system, as part of the non-adrenergic, non-cholinergic mechanism may have a role to play in contraction of the canine bladder.

In conclusion, the results of this study support the first hypothesis and demonstrate that the neutering of canines, regardless of gender, is associated with a reduction in the response of the detrusor muscle to non specific KCl, muscarinic and electrical field stimulation *in vitro*. This important observation has not been reported previously, as prior studies in rodents have only investigated the effects of neutering on females. It is hypothesized that the generalised decrease in responsiveness seen in neutered animals may be due to relative changes in urinary bladder wall structure, such as decreased smooth muscle and/or increased connective tissue. This study also demonstrates that there is a non-adrenergic, non-cholinergic mediated component of contraction in the canine. This non-adrenergic, non-cholinergic component appears to be mediated mainly via the purinergic system which is definitely not affected by gender nor gonadal status.

The changes seen to muscarinic and electrical field stimulation in neutered canines in this study may also be due to an, as yet, unidentified mechanism that involves the muscarinic receptor effector- pathway that could result in a condition similar to idiopathic detrusor instability and impaired contractility of the bladder in the human. Further factors that may interact with the receptor effector pathway are changes in sex hormone concentrations and steroid or gonadotrophin receptor expression in the bladder wall, that occur post neutering

(Reichler *et al.*, 2005b; Welle *et al.*, 2006). That the observed changes in KCl, muscarinic and electrical responsiveness are important factors for predisposing individuals to acquired urinary incontinence is supported by the data collected from neutered females that were identified as suffering from acquired urinary incontinence, as they showed the lowest contractility and responsiveness of the detrusor muscle. The muscarinic pathway may, therefore, present a potential new therapeutic target for treatment of this debilitating condition in the bitch, and an understanding of this pathway may help further our understanding of certain forms of the condition in the human model.

5 Morphometric Analysis of the Canine Urinary Bladder – The Effect of Neutering on Percentage Collagen

5.1 Introduction

As previously discussed, acquired urinary incontinence in the bitch is a debilitating and so far incurable condition. Acquired urinary incontinence is hypothesised to be hormonally related as it is reported to affect 20% of neutered bitches (Arnold *et al*., 1989) compared to 0-1% of intact bitches (Holt *et al*., 1993). Acquired urinary incontinence in the bitch is associated with a decrease in maximal urethral closure pressure (Rosin *et al*., 1981) but as this is not a defining characteristic, the exact pathophysiology of the condition remains unknown and multiple causative factors are likely to be involved. Urinary incontinence is also seen in post menopausal women where it is known that changes within the bladder wall are an important contributory factor to its development (Resnick *et al*., 1987).

Previous studies have reported that neutering of canines is associated with decreased bladder responsiveness to KCl and neurogenic field stimulation, and decreased sensitivity and responsiveness to muscarinic stimulation (chapter 4). These results are similar to those of women suffering urinary incontinence due to impaired contractility of the bladder and idiopathic detrusor instability (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b). These two conditions have been hypothesised to cause decreased strength of contraction of the bladder at the time of voluntary urination; as well as involuntary and unconscious partial contractions of the bladder during the storage phase of micturition. It has also been hypothesised that, in women, an increase in the collagen to smooth muscle ratio within the wall of the urinary bladder contributes to the pathophysiology of both idiopathic detrusor instability and impaired bladder contractility as it may physically change contractile ability and alter electrical conductivity within the smooth muscle that makes up the bladder wall (Charlton *et al.*, 1999; Chen *et al.*, 2002a).

The smooth muscle of the bladder wall is the component of the bladder that is directly responsible for bladder relaxation during the storage phase and bladder contraction during the expulsion phase of micturition. The detrusor muscle is made up of three layers of smooth muscle bundles arranged in an outer and inner longitudinal layer with a middle circular layer (Dyce *et al.*, 2002). The layers are made up of muscle bundles which in turn

are composed of muscle fibres grouped into fascicles or functional units (Dyce *et al.*, 2002). The smooth muscle of the bladder in the healthy individual contracts as a single unit via both gap junctions between the smooth muscle cells which allow direct electrical coupling, and via nervous coordination mediated by a dense nerve network (Andersson *et al.*, 2004a). In the human, it is known that a small amount of collagen is present within the bladder and that it is found within the connective tissue that surrounds the large muscle bundles as well as within the muscle bundles themselves (Andersson *et al.*, 2004a). If the amount of collagen increases in this area it can lead to problems with conductance of action potentials throughout the muscle layers due to disruption of the cell to cell contacts within the muscle bundles and could therefore affect micturition, potentially leading to urinary incontinence (Charlton *et al.*, 1999).

This study tested the hypothesis that, as in post menopausal women with urinary incontinence, the percentage collagen (relative to smooth muscle) within the urinary bladder wall will be increased in neutered compared to entire canines and that these changes will be most extreme in neutered bitches suffering from acquired urinary incontinence.

5.2 Materials and Methods

5.2.1 Animals

A total of 81 canines were included in the study, with a mean age of 6.0 years (range 1-14 years) and a mean weight of 25.6kg (range 8-48kg). The study was approved by The University of Glasgow Veterinary School's ethical review committee. The canines were split into five groups depending on gender, gonadal status and incidence of acquired urinary incontinence: entire and neutered males (ME, n=29 and MN, n=14), entire and neutered females (FE, n=25 and FN, n=11), plus neutered females known to be suffering from acquired urinary incontinence (FN AUI, n=3), as ascertained by history and physical examination. All the entire females in the study were considered to be in anoestrus, based on history and exam of the ovaries. All of the neutered canines used in this study were understood to have been neutered at least 6 months prior to euthanasia. To enable the best use of material obtained, a number of canines used in this study were also included in the tissue bath studies previously described. The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases, tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbatone), with full informed owner consent. In all cases, euthanasia occurred for reasons other than scientific investigation. The majority of animals were destroyed for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system except in the case of those animals known to be suffering from acquired urinary incontinence.

5.2.2 Preparation of Tissue

A full, detailed history of each animal was taken and a gross post-mortem of the entire urinary tract performed before the urinary bladder was harvested. The bladder was sectioned across the level of the ureters and a 2cm x 2cm full thickness section of the lateral dome removed and placed in 10% neutral buffered formalin (Bancroft *et al.*, 2003) for a minimum of twenty four hours. Any animal with a history of, or gross pathological signs of neurological, reproductive or urinary tract disease, other than acquired urinary incontinence, was excluded from the study.

5.2.3 Morphometric Analysis

Samples were transferred to an Excelsior Vacuum Tissue Processor (ThermoShandon, Thermo Fisher Scientific, UK) and processed for paraffin wax embedding using a standard 17 hour processing cycle. Samples were then embedded into paraffin blocks (Embedding Centre, Bayer, UK) and 5 micron sections cut on a Finesse microtome (ThermoShandon). Sections were transferred to a 60°C incubator for 60 minutes and deparaffinised in Xylene before being taken to water through graded alcohols. Sections were stained with Haematoxylin & Eosin and Masons Trichrome stain (Bancroft *et al.*, 2003), with the modification that Light Green was used instead of Methyl Blue and Orange G staining was added to allow visualisation of red blood cells.

Stained sections were evaluated for artefacts and staining qualities and two good quality sections (slices) from different areas of the tissue block were selected for detailed morphometric analysis. Morphometric evaluations were performed by light microscopic stereologic analysis, at a magnification of 40X, as described previously (Bartsch et al., 1979; Hashimoto et al., 1999; Shapiro et al., 1991). Briefly, 2-3 images were captured from each tissue slice (making a total of 5-6 images per animal) with a DM 4000 B microscope (Leica, UK). The percentage of collagen (stained green) relative to other tissue (all stained areas) within each field was then determined using image analyses software (FW 4000, Leica, UK). In this the picture was pixelated and colour segmentation used so that the green stained area could be accurately identified and quantified by the software using binary techniques. The green stained areas were calculated relative to all other stained areas, with any white areas within the field (such as produced by processing artefacts or the lumen of the bladder) discounted.

5.2.4 Statistical Analysis

A minimum of five fields over 2 tissue slices were evaluated for each animal and the results meaned to give the value for that animal. Data for each group (male, female, entire and neutered as well as neutered female animals known to be suffering from acquired urinary incontinence) are expressed as mean ± s.e.mean (n = number of canines).

Data were graphed and statistical analysis was performed using GraphPad Prism® v.5 software. Percentage collagen was compared between the 5 groups by one-way analysis of

variance (ANOVA) with Bonferroni post-test. A probability (P) less than or equal to 0.05 was considered statistically significant. Correlations between percentage of collagen and the age and weight of the animals, as well as between percentage of collagen and maximal response to carbachol (chapter 4), where appropriate, were assessed using Spearman's test with a significance threshold of P (two tailed) < 0.05.

5.3 Results

Staining with Masons Trichrome Orange G allowed visualisation of collagen (staining green) and smooth muscle (staining red) in all sections of bladder wall (Fig. 5-1). The percentage collagen in the bladder wall of entire canines was not affected by gender: ME 18.55 ± 0.99% (n=29), FE 17.45 ± 1.25% (n=25). The percentage of collagen within the bladder wall of the neutered males (19.18 ± 1.19%, n=14), was also not statistically different to that seen in the entire male and female canines. In stark contrast to the results seen in the males, the percentage collagen within the bladder wall in neutered females (42.94 ± 2.61%, n=11) was significantly increased ($P<0.001$) relative to that seen in their entire counterparts and indeed all other groups (Fig. 5-2). Further, the three neutered female canines known to suffering from acquired urinary incontinence had some of the highest values of percentage collagen within the study (54.3%, 58.7% and 48.7%), these values being above the 95% CI values (37.12 – 48.6) for the female neutered group. Visual inspection of the sections indicated that the greater percentage of collagen in the bladder wall of the neutered female animals was due to greater collagen deposition both between and within the muscle bundles. There was no correlation between age or weight and percentage of collagen within the urinary bladder wall.

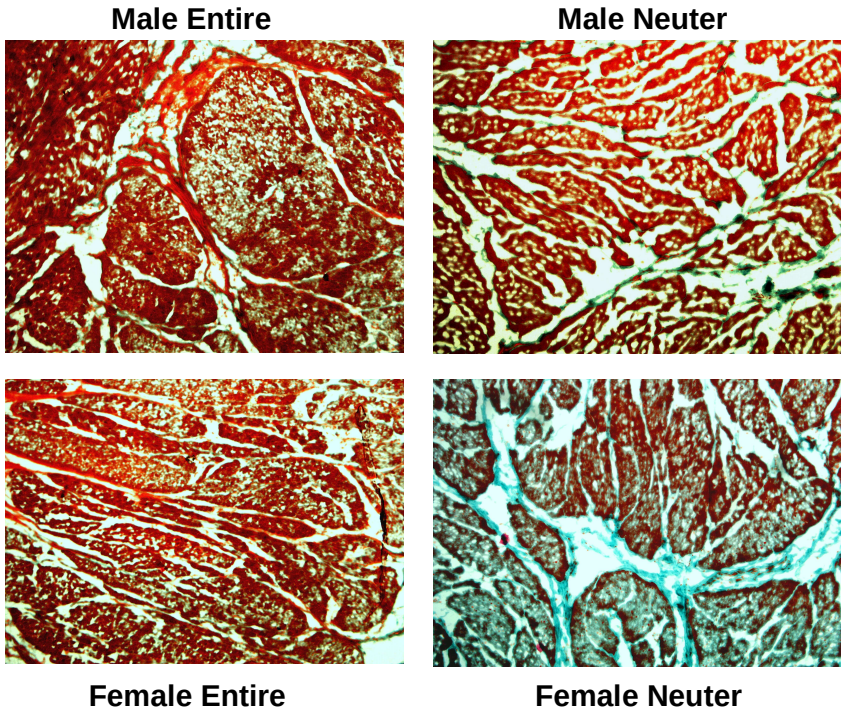

Figure 5-1. Representative sections of bladder wall (X40 magnification) from each group stained with Masson Trichrome Orange G to allow visualization of collagen (green) and smooth muscle (red).

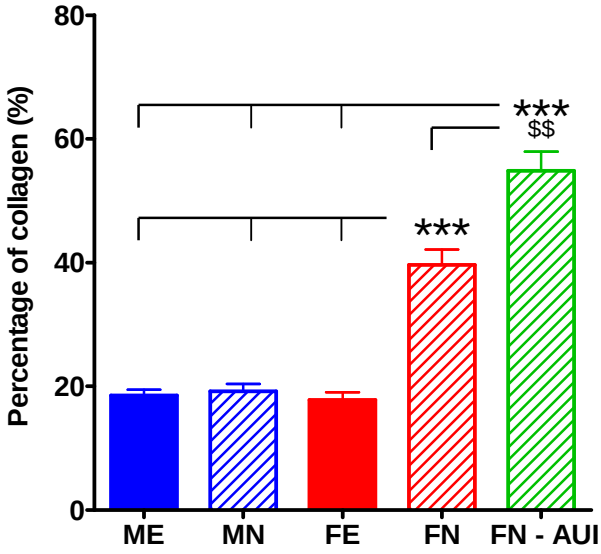

Figure 5-2. Summary data of percentage of collagen within the canine urinary bladder wall from entire and neutered male and female canines (ME n=29, MN n=14, FE n=25 and FN n=11 respectively), and neutered female canines suffering from acquired urinary incontinence (n=3). Data are mean ± s.e.mean. $$ P<0.05, *** P<0.001 compared to groups indicated.

Analysis of correlation between percentage collagen within the urinary bladder wall and maximal tension produced by isolated strips of detrusor muscle for each group did not reveal a statistically significant result, which may reflect the relatively low number of animals per group for which both sets of data were available. However, overall comparison of the maximal responses to carbachol and the percentage collagen within the urinary bladder wall indicated a statistically significant ($P<0.05$) negative correlation existed between these two characteristics (Fig. 5-3).

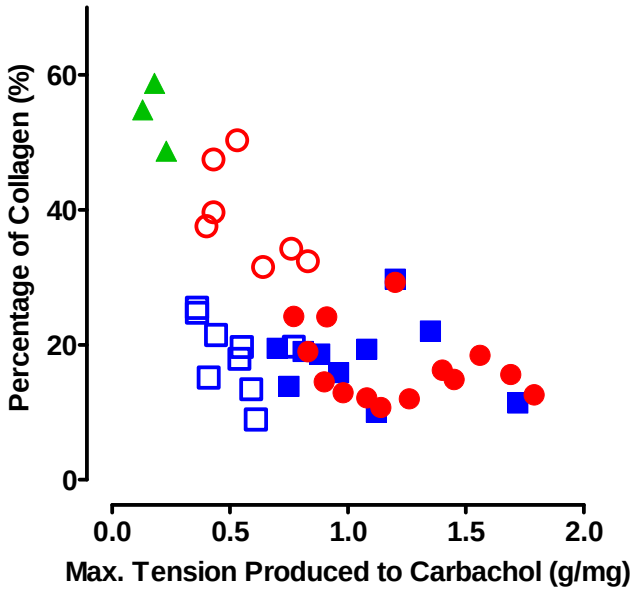

Figure 5-3. Graph showing overall negative correlation between maximal tension produced in response to carbachol stimulation against percentage collagen within the bladder wall ($P<0.05$), $r=-0.594$. ■ entire male, □ neutered male, ● entire female, ○ neutered female, ▲ neutered female known to be suffering from acquired urinary incontinence.

5.4 Discussion

Due to the observed relationship between percentage collagen in the bladder wall of post menopausal urinary incontinent women, relative to their continent peers, this study tested the hypothesis that percentage collagen (relative to smooth muscle) within the urinary bladder wall of neutered canines will be increased, compared to entire canines, with the changes being most extreme in neutered female canines that suffered from acquired urinary incontinence. The study included both male and female canines and, to the author's knowledge is the first study to look at the association between neutering and percentage collagen of the urinary bladder in a male animal of any species. The results demonstrate that the percentage of collagen within the urinary bladder of an entire canine is comparable between genders and interestingly that percentage collagen is only altered with gonadal status in females, in which a two-fold increase is seen, similar to that reported to occur in post menopausal human females who suffer from urinary incontinence (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b).

The increase in the percentage of collagen within the bladders of the neutered bitches in this present study is also similar to that reported in ovariectomised rodents, in which a decrease in relative smooth muscle content and an increase in percentage of collagen within the bladder wall has previously been reported (Fleischmann *et al.*, 2002; Zhu *et al.*, 2001). The results of this present study and those reported in the rat (Fleischmann *et al.*, 2002; Zhu *et al.*, 2001), however, are at odds with the results of a study conducted in rabbits which showed a relative decrease in the amount of connective tissue within the bladder after ovariectomy (Hashimoto *et al.*, 1999). While these differences in the post ovariectomy changes in percentage collagen in bladder wall structure could reflect species specific variation, it is important to note that there were also significant differences between the experimental designs used in these studies. Most notable of these, was the interval between ovariectomy and investigation, as the rabbit study reported acute (6 week) but the rat chronic (4 month), effects of ovariectomy on bladder wall structure. The changes noted in this canine study concur with those obtained in rats (Fleischmann *et al.*, 2002; Zhu *et al.*, 2001) and in that respect it is important to note that both studies reported chronic effects of ovariectomy. While the exact date of ovariectomy was not known for all of the animals in this present study population, based on anatomical features and the supplied history it can be reliably concluded that gonadectomy had occurred >6 months prior to study.

The observed increase in collagen within the bladder wall of the neutered females in this study appeared to occur both within and between the muscle bundles that make up the detrusor muscle. This location has potential negative functional consequences with regard to normal bladder function, as it may decrease the contractility and elasticity of the detrusor and / or affect bladder compliance. Both of these features may in turn affect the bladder's ability to relax and expand to store urine, a feature of the bladder which has been linked to urinary retention and urinary incontinence in the human (Chen *et al.*, 2002a). Intramuscular collagen deposition is also hypothesized to decrease conduction of action potentials throughout the muscle fascicle (Fleischmann *et al*., 2002), and thus could have a negative effect on the ability of the bladder to contract as a single functional unit. The possibility that the observed changes in collagen deposition may predispose neutered bitches to acquired urinary incontinence is supported by the data from the neutered females known to be suffering from acquired urinary incontinence which had some of the highest percentage collagen of all the animals studied, but as shown in chapter 4, the lowest responses to KCl, muscarinic and electrical field stimulation of all animals tested. An increase in percentage collagen has also been reported in the wall of the urinary bladder of women with detrusor instability, where it is associated with an altered sensory threshold for cholinergic stimulation of the bladder (Charlton *et al.*, 1999), which leads to decreased bladder filling and increased partial bladder contractions, often out-with the conscious control of the patient.

The changes in muscle structure and percentage collagen described above have been proposed to be brought about by the changes in female reproductive hormones, namely a loss of oestrogen, that occur after the menopause (Thom *et al.*, 1998) or ovariectomy (Thrusfield, 1985). This conclusion is supported by data from the rat and rabbit where smooth muscle fibres were demonstrated to be maintained, and in some cases increased, by exogenous oestrogen treatment of ovariectomised animals (Fleischmann *et al.*, 2002; Hashimoto *et al.*, 1999; Zhu *et al.*, 2001). In addition, a study that looked at the coronary vessels of female rats has also demonstrated that oestrogen has a protective effect on these structures, following ovariectomy, as it prevents the proliferation of fibroblasts and the deposition of collagen within the smooth muscle of the vessel walls (Blacher *et al.*, 2000). Despite these reported protective effects of oestrogen on smooth muscle, there are no studies that document that exogenous oestrogen supplementation can prevent changes occurring after the discontinuation of treatment, as was hoped with hormone replacement therapy (HRT) or reverse changes that have already occurred. Therefore, oestrogen supplementation after the appearance of clinical signs of urinary incontinence has developed is likely to be too late to reverse the structural changes within the bladder that

might accompany urinary incontinence. Indeed, this may explain why replacement oestrogen therapy, a standard treatment for urinary incontinence in the bitch, whilst known to increase the urethral closure pressure in incontinent bitches (Creed, 1983), only produces improvement in continence in approximately 65% of bitches (Arnold *et al.*, 1989; Janszen *et al.*, 1997; Janszen BPM, 1997; Mandigers *et al.*, 2001) and often only temporarily (Hotston Moore, 2001).

This lack of efficacy of supplemental exogenous oestrogen, as a cure for acquired urinary incontinence in the bitch, is not necessarily the calamity it was once thought, however, as other studies that have looked at the effects of gonadectomy on smooth muscle structure and function (Fleischmann *et al.*, 2002; Hashimoto *et al.*, 1999; Zhu *et al.*, 2001) have concentrated on the female and have not looked at the effects on the male of the species. These studies, therefore, may have concluded a potentially greater role for the loss of oestrogen than may be warranted. The gender specific effect of neutering on the percentage of collagen within the urinary bladder that this present study has demonstrated is significant, as earlier work has shown that there is a significant decrease in contractility of the detrusor muscle to KCl, muscarinic and electrical field stimulation, and a decrease in sensitivity to muscarinic stimulation, in neutered animals of both genders (chapter 4). Previous studies in postmenopausal women and ovariectomised rodents have found similar decreases in function of the detrusor muscle, and the authors of these studies have related these changes to increases in the collagen to smooth muscle ratio within the bladders studied (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b; Fleischmann *et al.*, 2002). This present study, the first to include male animals, shows clearly that the decrease in function following gonadectomy is evident in both males and females, but that the increase in the percentage of collagen is only evident in the bladders of the neutered female animals. This suggests that a further mechanism is involved in the decrease in contractile function of the bladder in gonadectomised animals and this may involve the muscarinic receptor effector pathway which may in turn be influenced by the changes in gonadotrophin hormone concentrations post neutering, as well as other, as yet unidentified factors.

In conclusion, this study demonstrates that neutering a bitch significantly increases the percentage of collagen within the urinary bladder wall, whilst neutering a male canine has no effect on percentage of collagen when compared to entire animals of either gender. The results from the neutered bitches that were known to be suffering from acquired urinary incontinence show the highest percentage collagen of all animals studied and suggest that this increase may have a role to play in the development of clinical signs of urinary incontinence; however, the significance of this interaction remains unknown. The

difference in the effects of gonadectomy on percentage collagen suggests that the role of collagen in altering the contractility of the bladder may have been overemphasised previously and with the previous *in vitro* contractility study, suggests that further mechanisms are involved in regulating the decreased responsiveness of the detrusor muscle post neutering in both genders. For example, changes in reproductive hormone levels, their receptors and signalling mechanisms or in the muscarinic effector pathway within the bladder must be investigated to allow clearer understanding of the aetiology and pathophysiology of both acquired urinary incontinence in the bitch and postmenopausal incontinence in women.

6 Pharmacological Characterisation of Muscarinic Receptors in Canine Urinary Bladder

6.1 Introduction

Acquired urinary incontinence in the bitch is a widespread problem causing significant welfare complications for the animals affected. Acquired urinary incontinence is currently reported to affect up to 20% of all neutered bitches (Arnold *et al.*, 1989), whilst less than 1% of entire bitches and males are thought to suffer from the condition (Holt *et al.*, 1986). Although a decrease in resting tone within the urethra has long been proposed as the primary mechanism by which acquired urinary incontinence occurs (Holt, 1988), further mechanisms are thought to have a role to play in the aetiology and pathogenesis of the condition. In post menopausal women who suffer from urinary incontinence, a group thought to share a similar aetiology to that of the canine disease, the development of urinary incontinence is known to involve changes within the urinary bladder, including structural alterations and responsiveness to muscarinic stimulation (Elbadawi *et al.*, 1993a; Resnick *et al.*, 1987).

The results of chapter 4 demonstrated that there is a significant decrease in *in vitro* sensitivity and maximal contractility in response to muscarinic stimulation of isolated strips of detrusor muscle in neutered compared to entire animals, regardless of gender. Furthermore I have demonstrated that these changes cannot be solely due to structural alterations within the bladder, as the percentage collagen within the bladder only increased in neutered female and not male animals (chapter 5). This suggests that there is a common structural pathway or molecular target in both genders that is affected by neutering and has a role to play in the altered contractility of the bladder. Given the above differences in response to muscarinic stimulation and as the muscarinic pathway is known to be the primary pathway responsible for micturition (Chess-Williams, 2002) it follows that changes within it may alter the contractility of the bladder.

Studies in a number of animals have shown that all five muscarinic subtypes are present within the urinary bladder (Abrams *et al.*, 2006; Chess-Williams, 2002; Wang *et al.*, 1995), but that the M_2 and M_3 receptors predominate, with the M_2 receptor out numbering the M_3 receptor subtype (Hegde *et al.*, 1999; Wang *et al.*, 1995). Despite this, it is the M_3 receptors that are thought to be the principle receptors responsible for regulating micturition and bladder contraction in healthy individuals, including humans (Chess-

Williams, 2002), rats (Longhurst *et al.*, 2000), guinea-pigs (Wang *et al.*, 1995) and canines (Choppin *et al.*, 2001). Interestingly, however, a study using M_2, M_3 and M_2/M_3 receptor knockout mice has shown that the M_2 receptor can not only enhance the contractile response to M_3 activation but also directly stimulate detrusor contraction (Ehlert *et al.*, 2005). This role for the M_2 receptor in bladder contraction is further strengthened by a recent study in rats that indicated a minor role for both the M_1 and M_2 receptor subtypes in urinary bladder contraction and relaxation (Frazier *et al.*, 2007). Of particular interest with regard to an effect of receptor isoforms and possible causes of altered bladder function post-neutering or after alterations in hormone levels, are the reports that the proportions of muscarinic receptor subtypes within the urinary bladder may be altered in terms of density and function in different disease / physiological states (Abrams *et al.*, 2006; Chess-Williams, 2002). In particular, it has been shown that following denervation of the bladder, the role of the M_3 receptor is diminished and the M_2 subtype becomes the predominate receptor responsible for bladder contraction (Braverman *et al.*, 1998b; Pontari *et al.*, 2004). This is potentially significant, with regard to acquired urinary incontinence in the bitch, as a limited study in pigs has suggested that bladder contractions mediated via stimulation of the M_2 receptor may be weaker than those mediated by the M_3 receptor and that the sensitivity of the M_2 receptor to carbachol may be less than that of the M_3 receptor (Yamanishi *et al.*, 2000).

The above findings lead to the possibility that the altered contractility of the detrusor muscle demonstrated in neutered canines (chapter 4) may be influenced by a change in the functional muscarinic receptor subtype in the bladder. This study will thus test the hypothesis that neutering will alter the functional muscarinic receptor subtype of the bladder from predominately the M_3 to the M_2 receptor.

6.2 Materials and Methods

6.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissues from a total of 15 canines were included in the study, although not all animals were included in each protocol. The study population had a mean age of 4.8 ± 1.2 years (range 1-12 years) and a mean weight of 24.2 ± 3.7kg (range 9-45kg). The canines were split into two groups depending on gonadal status. The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbitone), with full informed owner consent, for reasons other than scientific investigation. The majority of animals had been euthanized for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. In all cases a detailed history of each animal was taken and a gross post-mortem study of the entire urinary system was performed; any animals with a history of, or gross pathological signs of, urinary tract disease were excluded from the study.

6.2.2 Preparation of Tissue

For all tissue bath protocols, bladder tissue was used within 2 days of euthanasia of the animal (see chapter 3). To maximise the use of tissues, a number of the animals included in this study were also included in the studies described in elsewhere in this thesis.

Preparation of detrusor strips was as for chapter 4, with muscle strips being mounted on fixed hooks for carbachol concentration response protocols (6.2.4).

Before addition of the muscarinic antagonists, strips were tensioned until stable at 4g tension and were re-tensioned as required throughout the incubation period. During incubation procedures the isolated strips of detrusor muscle were maintained in the tissue baths at 37°C, bathed in standard Krebs solution (chapter 3), with the addition of the muscarinic antagonist in question and continually aerated with 95% O_2 / 5% CO_2.

6.2.3 Muscarinic Antagonist Preparations

Pirenzepine (Sigma-Aldrich, UK), a selective M_1 receptor antagonist (Caulfield *et al.*, 1998), was diluted in water (BDH, RNase and DNase free) to give a stock solution of 10mM which was stored at -20°C. On the day of each experiment, the stock solution was diluted in Krebs solution and added to each tissue bath, as described below, to give a final bath concentration of 10nM, 30nM or 100nM (these being the optimal concentrations, as determined by preliminary studies to determine the lowest concentration range of antagonist required to produced a right shift in the response to carbachol – data not shown).

Methoctramine (Sigma-Aldrich, UK), an M_2 selective antagonist (Caulfield *et al.*, 1998), was diluted in water (BDH, RNase and DNase free) to give a stock solution of 10mM which was stored at -20°C. On the day of each experiment, the stock solution was diluted in Krebs solution and added to each tissue bath, as described below, to give a final bath concentration of 100nM, 300nM or 1µM (these being the optimal concentrations, as determined by preliminary studies to determine the lowest concentration range of antagonist required to produced a right shift in the response to carbachol – data not shown).

4-diphenyl-acetoxy-*N*-methyl piperidine methiodide (4-DAMP) (Tocris Bioscience, Bristol, UK), an M_3 selective antagonist (Caulfield *et al.*, 1998), was diluted in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, UK) to give a stock solution of 100mM which was stored at -20°C. On the day of each experiment, the stock solution was diluted in Krebs solution and added to each tissue bath, as described below, to give a final bath concentration of 1nM, 3nM or 10nM (these being the optimal concentrations, as determined by preliminary studies to determine the lowest concentration range of antagonist required to produced a right shift in the response to carbachol – data not shown).

6.2.4 Carbachol Concentration Response Protocol with Muscarinic Antagonists

All strips (~ 8 per animal) underwent a full carbachol concentration response protocol, the full results of which are incorporated into the data presented in chapter 4.

Following completion of the initial carbachol concentration response protocol, the muscle strips were washed repeatedly, allowed to rest for 30 minutes and the tension adjusted until

stable at 4g resting tension. Pirenzepine, Methoctramine and 4-DAMP were then added to two baths each, to give the initial bath concentration for that antagonist described above, leaving two of the eight strips without any additional antagonist, to act as time controls. Strips were incubated for 30 minutes, after which time they were re-tensioned to 4g initial tension (as necessary) before a further full carbachol concentration response protocol was carried out in all eight strips. This protocol of washing, re-tensioning, addition of muscarinic antagonist, incubation and full carbachol concentration response protocol was repeated as described above for all three concentrations of each antagonist.

6.2.5 Data Analysis

Data were normalised for wet weight of tissue and are expressed as g/mg of wet tissue. Results are presented as mean ± s.e.mean (n = number of animals).

A minimum of 2 strips of bladder tissue per animal were analysed for each muscarinic antagonist, at each concentration and the mean results from these strips used in further calculations where applicable. Data were graphed and statistically analysed using GraphPad Prism® v.5 software. EC_{50} values (molar concentration producing half maximal response) were determined by non-linear regression fitting (variable slope) to the concentration response curves. Data from curves from individual animals were used to derive mean –log EC_{50} (pEC_{50}) values with s.e.mean. Dissociation constants (pK_B) for antagonists were determined from the equation:

$$pK_B = \log(CR-1)-\log[B]$$

where CR is the concentration ratio (ratio of the EC_{50} values) in the absence and presence of the antagonist obtained with a concentration [B] of antagonist (Furchgott, 1972). Schild analysis was also performed, and the resultant gradient used to assess the competitive nature of the antagonism (Arunlakshana *et al.*, 1959).

Differences in pEC_{50} and maximum responses, between groups, were determined by analysis of variance (ANOVA) with Bonferroni as a post-test. A probability (P) less than or equal to 0.05 was considered significant. Correlations between responses and the age and weight of the animals were conducted using Spearman's test with a significance threshold for P (two tailed) < 0.05.

6.3 Results

As previously reported (chapters 3 and 4), carbachol induced concentration-dependant contractions of isolated strips of smooth muscle from canine urinary bladder. Strips of bladder tissue from neutered animals had significantly lower maximal contractile responses ($p<0.05$) and significantly decreased sensitivity compared to tissue from entire counterparts ($p<0.05$), as previously shown in chapter 4. Analysis of the control strips which received four consecutive carbachol concentration response curves, i.e. a time control study, indicated that potency and maximal response were not altered by repeated testing (Fig. 6.1).

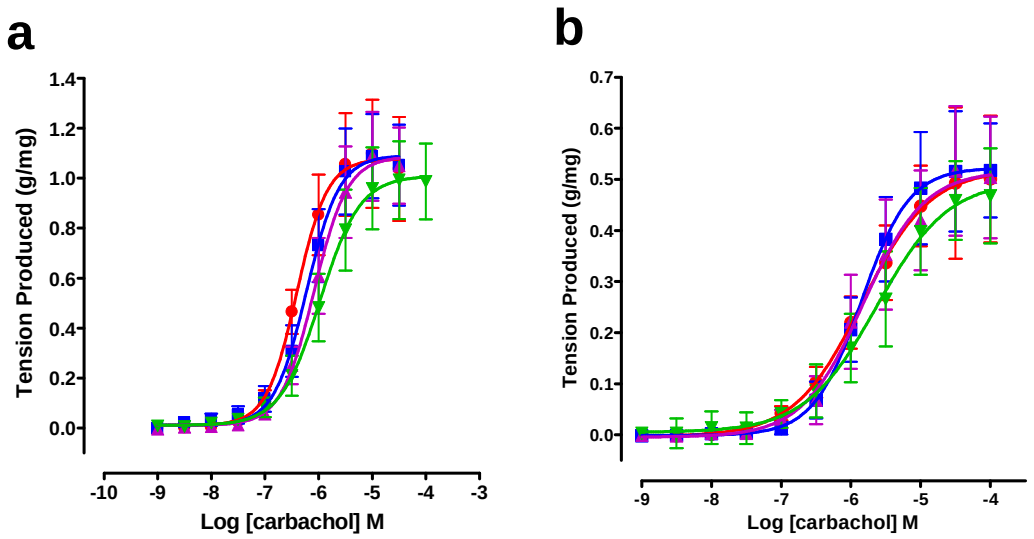

Figure 6-1. Cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips over time. Each point is the mean ± s.e.mean of observations from n animals. ● Time 0 hours, ■ Time 1.5 hours, ▲ Time 3 hours, ▼ Time 4.5hrs. a, entire animals, n=9; b, neutered animals, n=6.

In all tissues the M_1 selective antagonist pirenzepine, the M_2 selective antagonist methoctramine, and the M_3 selective antagonist 4-DAMP acted as competitive antagonists of carbachol-induced contractile responses. All antagonists produced parallel rightward shifts of the concentration-response curves to carbachol, without altering maximal responses (Fig. 6-2). When Schild plots were calculated, however, only methoctramine and 4-DAMP produced slopes not significantly different from unity (Fig. 6-3). In all cases, due to the low numbers of animals studied and the mixed gender groups the s.e.mean is greater than that for previous chapters.

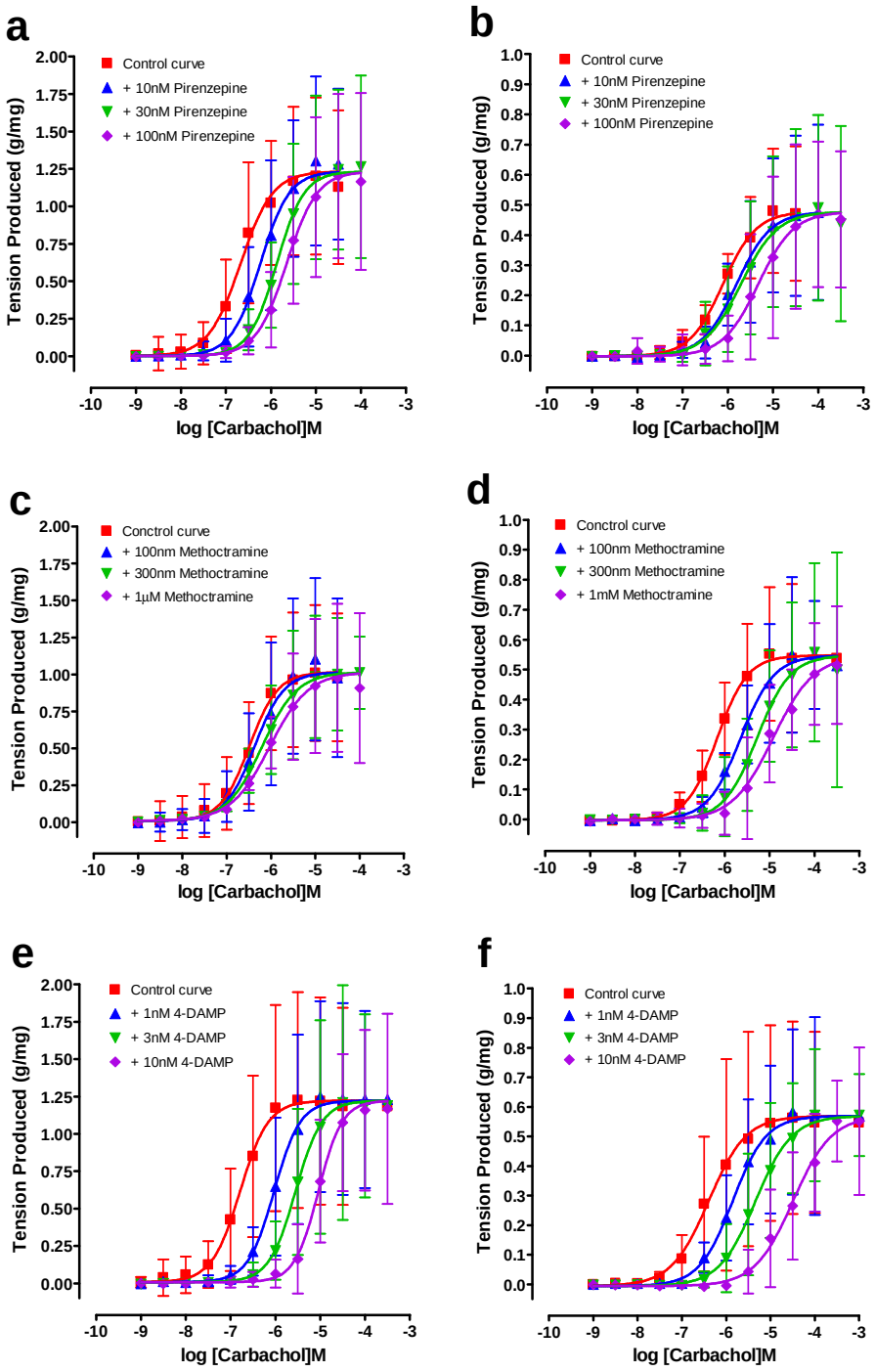

Figure 6-2. Effects of Pirenzepine, Methoctramine and 4-DAMP on cumulative concentration response curves of carbachol in isolated strips of canine urinary bladder smooth muscle strips. Each point is the s.e.mean of observations from n animals. a, c and e are from entire animals, n=9; b, d and f are from neutered animals, n=6.

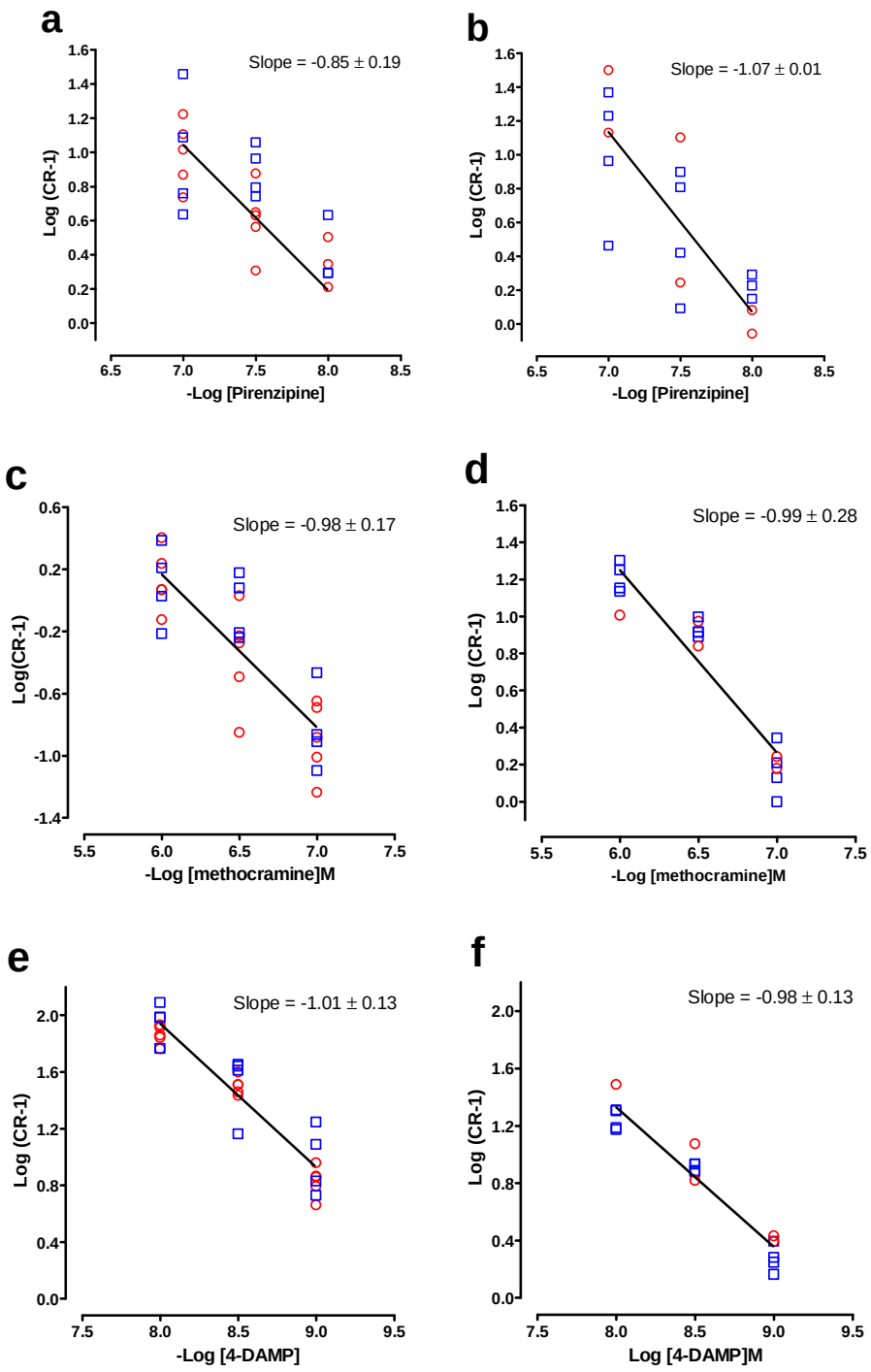

Figure 6-3. Schild plots for antagonism of the carbachol response by Pirenzepine, Methoctramine and 4-DAMP in isolated strips of detrusor muscle. Each point represents the mean results from an individual animal at each concentration of antagonist. a, c and e are in entire animals; b, d and f are in neutered animals. ○ Female canines, □ Male canines.

To ensure the accuracy of the mean functional affinity estimate (pK_B) for each antagonist, the pK_B at each antagonist concentration was calculated (Table 6-1). The rank order of antagonist affinities in the entire group of canines was methoctramine, pirenzepine and 4-DAMP, and in the neutered group of canines was pirenzepine, methoctramine and 4-DAMP.

Antagonist	**Concentration**	**pK_B - Entire**	**pK_B - Neutered**
	10nM	8.14 ± 0.14	8.20 ± 0.04
Pirenzepine	30nM	7.52 ± 0.17	7.84 ± 0.04
	100nM	8.00 ± 0.09	8.19 ± 0.09
	100nM	7.04 ± 0.08	8.19 ± 0.05
Methoctramine	300nM	6.74 ± 0.13	7.93 ± 0.06
	1µM	7.12 ± 0.07	8.18 ± 0.12
	1 nM	9.38 ± 0.06	8.29 ± 0.03
4-DAMP	3 nM	8.99 ± 0.05	7.92 ± 0.03
	10 nM	9.40 ± 0.04	8.28 ± 0.05

Table 6-1. Affinity estimates (pK_B) at each concentration for muscarinic antagonists in bladder smooth muscle from entire and neutered canines.

As Schild plots did not give a slope of unity for pirenzepine the antagonist affinities obtained cannot accurately be correlated with the known muscarinic receptor subtype ranges, however for both methoctramine and 4-DAMP the slopes of unity obtained from Schild analysis suggests that there is binding at a single muscarinic receptor subtype. For entire canines the overall affinity of both methoctramine and 4-DAMP was within the reported range for the M_3 subtype, whilst in neutered canines the overall affinities were consistent with the M_2 subtype (Table 6-2).

Antagonist	M_1	M_2	M_3	M_4	M_5	Entire Bladder	Neutered Bladder
Pirenzepine	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	6.2-7.1	n / a	n / a
Methoctramine	7.1-7.8	7.8-8.3	6.3-6.9	7.4-8.1	6.9-7.2	6.90 ± 0.13	8.10 ± 0.09
4-DAMP	8.6-9.2	7.8-8.4	8.9-9.3	8.4-9.4	8.9-9.0	9.06 ± 0.13	8.16 ± 0.12

Table 6-2. Antagonist affinity constants (log affinity constants of pK_B values) for mammalian muscarinic receptors and for bladder smooth muscle from entire and neutered canines. Data are from a variety of mammalian species (Caulfield *et al.*, 1998).

Looking at individual animals within each group it is possible to see that five animals within the entire group have pK_B values that are intermediate between the M_2 and the M_3 receptor ranges in response to either methoctramine or 4-DAMP (Fig. 6-4), however no animal was intermediate for both antagonists. When looking at individual animals in the neutered group only one animal has a pK_B value that is intermediate between M_2 and M_3 receptors, and that is only in response to methoctramine.

Although the number of male and female animals in each group differed and was not large enough to allow detailed statistical analysis there did not appear to be an effect of gender on the results obtained within each group. In addition statistical analysis demonstrated that there was no effect of age or weight on the resultant antagonist affinities.

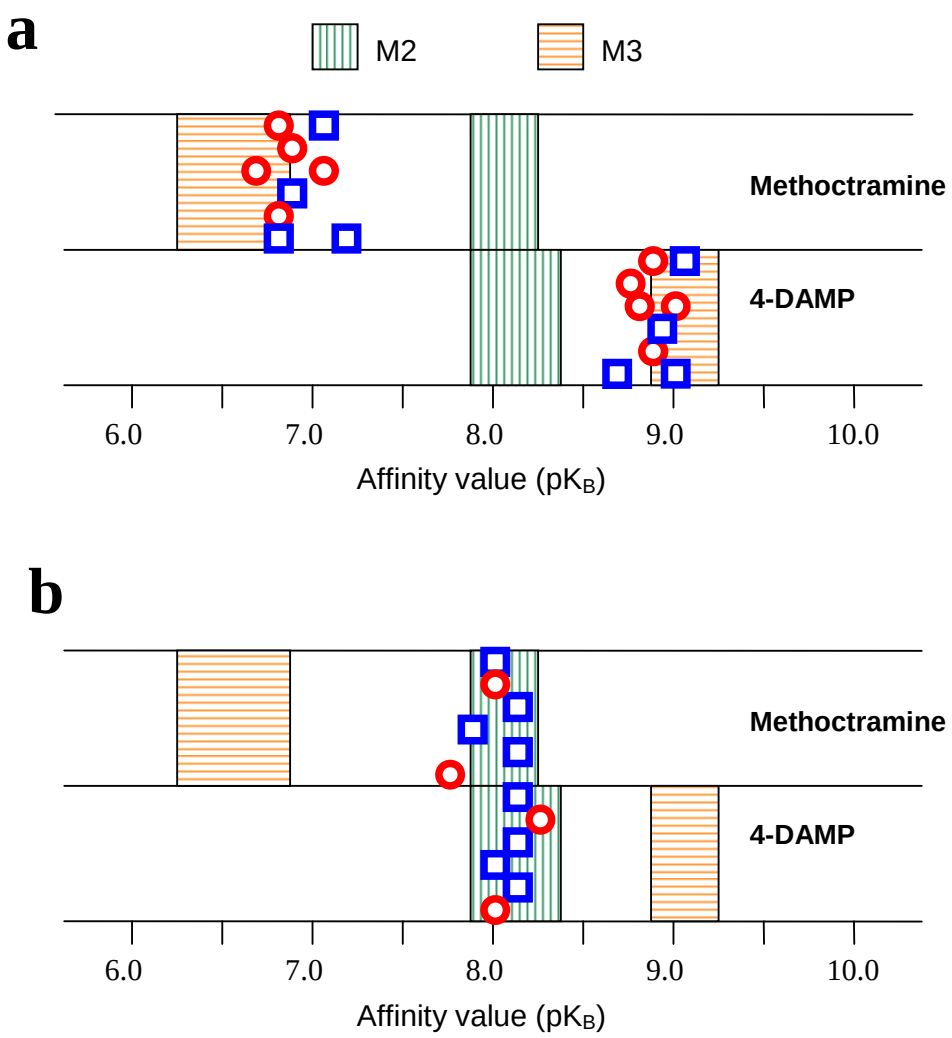

Figure 6-4. Affinity of subtype-selective antimuscarinics for inhibiting carbachol induced contraction of strips of urinary bladder smooth muscle *in vitro* from a, entire and b, neutered canines. The shaded areas represent the affinity ranges of methoctramine and 4-DAMP for the individual receptor subtypes reported in the literature (Caulfield et al., 1998). Symbols refer to the affinities that were determined by Schild analysis of the bladder tissue obtained from individual animals. ○ Female canines, □ Male canines.

6.4 Discussion

It is thought that detrusor muscle contractions in healthy individuals are mediated via the M_3 receptor subtype. This is based on *in vitro* evidence from a number of species including rat (Hegde *et al.*, 1997), pig (Sellers *et al.*, 2000), human (Chess-Williams *et al.*, 2001) and canine (Choppin *et al.*, 2001). There is also evidence from rats and humans, that in certain disease states, the muscarinic receptor subtype responsible for bladder contraction can change from the M_3 to the M_2 receptor subtype (Braverman *et al.*, 1998b; Pontari *et al.*, 2004). This present study, the first to include neutered canines, confirms previous reports (Choppin *et al.*, 2001) that contraction of the canine urinary detrusor in entire animals is mediated by the M_3 receptor, but clearly demonstrates that in the neutered canine contraction of the urinary detrusor appears to be mediated via stimulation of the M_2 rather than the M_3 receptor subtype.

In all of the animals studied carbachol produced concentration dependant contraction of the isolated strips of detrusor muscle, as has been previously discussed, and the maximal tensions produced where similar to those described in chapter 4 of this thesis. Despite this, the calculated s.e.mean for each group of animals was larger than that previously recorded. This may be due to a number of reasons including the relatively low numbers of animals included in each group, and the low number of muscle strips from each animal included for each antagonist studied. A further factor to be considered is that due to low animal numbers for this study both male and female animals were included in the entire and neutered groups, thereby increasing the error. Although male and females animals did not vary significantly in their response to carbachol (see chapter 4) there was a trend for the male animals to have lower responses than their female counterparts, and due to individual variation and numbers of animals used this is likely to account for the increased s.e.mean seen.

Bladder contraction occurs from ACh-induced excitation of muscarinic cholinergic receptors on detrusor muscle (Chess-Williams, 2002). There are known to be five muscarinic receptor subtypes, M_1-M_5, however only the first three have been consistently described as having a role in micturition (Braverman *et al.*, 1998a; Chess-Williams, 2002; Somogyi *et al.*, 1994). Subtype selective antimuscarinic agents are available for the M_1-M_3 subtypes that are at least 10-fold selective for individual subtypes allowing *in vitro* studies to identify functional receptors within a tissue (Caulfield, 1993). M_1 receptors have a high affinity for pirenzepine, a low affinity for methoctramine and an intermediate

affinity for 4-DAMP (Caulfield *et al.*, 1998). M_2 receptors have a high affinity for methoctramine and a low affinity for 4-DAMP, whilst it is the opposite for M_3 receptors which have a high affinity for 4-DAMP and a low affinity for methoctramine (Caulfield, 1993). Using these agents and calculating affinity values derived from Schild plot analysis, it has been shown that the M_3 receptor is responsible for the contraction of the healthy human bladder, even though the number of M_2 receptors far exceeds the number of M_3 receptors (Chess-Williams *et al.*, 2001). This is consistent with results in other animal species that report that although the M_2 receptor subtype is the most numerous, within the urinary bladder, it is the M_3 receptor that is responsible for normal bladder contraction (Chess-Williams, 2002; Goepel *et al.*, 1998; Wang *et al.*, 1995).

Studies into the role of the M_1 receptor in the rat have shown that this receptor subtype may have a facilitatory role to play in contraction of the bladder by enhancing ACh release thereby enhancing contraction of the bladder (Braverman *et al.*, 1998a; Somogyi *et al.*, 1994). In this present study, Schild plot analysis did not demonstrate a slope of unity for pirenzepine antagonism of carbachol induced contractions in either entire or neutered canine bladder, however, the reliability of the data is limited by the low number of animals studied. The affinity values of the neutered animals in response to pirenzepine all fell within the reported range for the M_1 receptor (Caulfield *et al.*, 1998), therefore it is possible that the M_1 receptor subtype has a facilitatory role to play in the regulation of detrusor contraction in this subset of canines and this hypothesis requires further investigation. For the entire canines the Schild plot gave a slop of less than 1, which can indicate that more than one receptor subtype is involved in mediating the contractile response (Wyllie *et al.*, 2007). It is interesting to note that the affinity values calculated for the gonadally entire group fell between the reported values for the M_1 and M_3 receptors (Caulfield *et al.*, 1998). This may indicate that both of the M_1 and M_3 receptor subtypes have a role to play in bladder contraction in this group.

The results obtained in this study, using both methoctramine and 4-DAMP, confirm that the M3 receptor subtype is the primary receptor subtype responsible for bladder contraction in healthy gonadally entire canines (Chess-Williams 2002). These results also are in agreement with a previous, limited, study by Choppin and Eglen which investigated functional receptor subtypes in the bladder using the muscarinic antagonists s-secoverine and darifenacin (Choppin *et al.*, 2001). In that study the affinities of the antagonists for inhibition of bladder contraction were shown to be correlated with their affinities for each of the muscarinic subtypes, expressed in clonal cell lines and it was assumed that the subtype that gave the highest correlation coefficient mediated bladder contraction. Their

method gives the same weighting to antagonists of varying or no specificity and also assumes that only a single muscarinic receptor subtype mediates contraction. In the present study, the individual affinity values for each antagonist, in each animal were calculated, as this allows for more detailed analysis both within and between groups of animals and therefore removes the constraint of the assumption that only one receptor subtype is involved in contraction. This is important, as previous studies have shown that more than one muscarinic receptor subtype is indeed involved in bladder contraction, either via indirect mechanisms that may influence neuronal acetylcholine release (Chess-Williams, 2002) or altered calcium flow (Wang *et al.*, 1995). The results of the present study show that although overall the M_3 receptor seems to be responsible for bladder contraction, if individual canine data is considered there are a number of animals with intermediate affinity values that fall between the reported values for the M_2 and M_3 receptors to either methoctramine or 4-DAMP. This result would suggest that there may also be a small role for the M_2 receptor in bladder contraction in some entire animals. This result supports those obtained in a study conducted using samples of human bladder that also concluded that in certain patients bladder contraction could be mediated by either the M_2 or the M_3 receptor (Pontari *et al.*, 2004) and a study in rats that reported that both M_2 and M_3 could mediate contraction in the normal rat bladder (Braverman *et al.*, 2002).

A role for the M_2 receptor subtype in mediating bladder contraction in healthy individuals is further supported by rodent studies that identified both M_2 and M_1 receptors as being involved in bladder contraction (Frazier *et al.*, 2007; Stengel *et al.*, 2000). Although this present study did not find a conclusive role for the M_2 receptor in entire canine bladder contraction, the overall results for both methoctramine and 4-DAMP in the group of neutered canines supports a major role for the M_2 receptor in this subset of animals. Of the six animals studied only one animal had a calculated pK_B value out-with that accepted as associated with the M_2 receptor and that was only for the antagonist methoctramine; when the antagonist 4-DAMP was used, the calculated pK_B value for that animal was consistent with the M_2 receptor. Although only a small number of animals were included in this study the consistency of the results, obtained to date, suggest that the M_2 receptor is involved in bladder contraction in gonadectomised canines. As this is the first study to include neutered canines this result is significant and may be linked to the decreased overall contractile response of the detrusor muscle *in vitro* in this group.

There have been several hypothesized mechanisms through which M_2 receptors may produce bladder contraction. Studies looking at contraction of the rat urinary bladder after alkylation of M_3 receptors with 4-DAMP mustard suggest that M_2 receptors inhibit β-

adrenergic receptor induced relaxation, via activation of a GTP binding protein that inhibits adenylyl cyclase (Braverman *et al.*, 1999b; Hegde *et al.*, 1997). While muscarinic receptor subtypes have been shown to preferentially couple with one type of G protein, using cloned receptors expressed in Chinese hamster ovary (CHO) cells, there does seem to be significant promiscuity in this coupling mechanism (Tucek *et al.*, 2002). This means that depending on the cell type and the biochemical state of the cell, certain receptors may be able to couple with several different types of G protein and therefore that a single receptor subtype may be able to mediate a number of cellular signals (Tucek *et al.*, 2002; Wang *et al.*, 1995). Traditionally M_2 receptors have been thought to couple with the G_i class of GTP binding proteins, however, it has been shown in human bladder (Wang *et al.*, 1995) that they can also couple to G_q proteins. It follows, therefore, that differential coupling of M_2 receptors to different G proteins, under varying conditions, may alter their effect on bladder tissue. In other organs that contain smooth muscle, a number of other mechanisms of M_2 receptor activation have been described and these may also have a role to play in the bladder. These include the opening of non-selective cation channels which results in depolarisation, the influx of calcium and contraction of the cells (Bolton *et al.*, 1997), and the inhibition of conductance of action potentials through potassium channels (Cole *et al.*, 1989).

Despite the various hypotheses surrounding the mode of action of the M_2 receptor subtype in mediating contraction of the bladder, there are conflicting reports as to its role in specific disease states. Neurogenic overactivity of the bladder has been studied in both the rat model and in the human, with inconsistent results. One group has reported that the M_2 receptor contributes to direct detrusor contraction in the urinary bladder obstructed rat and in the spinal injured, denervated, non-voiding rat (Braverman *et al.*, 1999a; Braverman *et al.*, 1998b; Braverman *et al.*, 2003; Ruggieri *et al.*, 2006), whilst another group could find no evidence of a role for the M_2 receptor subtype in stimulating bladder contraction in the urinary bladder obstructed rat (Krichevsky *et al.*, 1999). In human patients with neurogenic overactivity of the bladder there are also conflicting reports, with one group concluding that the M_2 receptor plays a role in bladder contraction in these patients (Pontari *et al.*, 2004), and a further group finding no evidence to support a role for M_2 and concluding that only the M_3 receptor was responsible for bladder contraction in this patient group (Stevens *et al.*, 2007). One study has been reported, that looked at the role of muscarinic receptors in patients (n=7) that were suffering from idiopathic detrusor overactivity (Stevens *et al.*, 2007). They reported that the M_3 receptor was the sole receptor responsible for bladder contraction in this group (Stevens *et al.*, 2007). These conflicting results, between studies and conditions, could be due to a number of factors

including experimental design, low patient numbers, varying severity of disease and individual animal and patient variations, especially involving the degree of detrusor muscle hypertrophy, a factor that has been shown to be highly significant in the rat model (Ruggieri *et al.*, 2006).

The functional significance of a shift from M_3 receptor mediated contraction to M_2 mediated contraction in the canine bladder as described in the present study has not been determined. It has been shown, in chapter 4, that neutered canines have significantly lower maximal contractile responses and sensitivity to carbachol than their entire counterparts, regardless of gender, and the present study shows that contraction of the detrusor is mediated by the M_2 receptor subtype in at least a subset of the same group of animals. It may be that the decreased response to carbachol observed in neutered animals (chapter 4) is directly linked to this shift in dependence on the M_2 receptor, as bladder contractions mediated by the M_2 receptor subtype have been reported to be weaker than those induced by the M_3 receptor subtype, at least in pigs (Yamanishi *et al.*, 2000). The sensitivity of the M_2 receptor subtype to carbachol is controversial, however, as some studies in rats and humans have found an increased sensitivity of the bladder to carbachol in models of neurogenic detrusor overactivity (Braverman *et al.*, 1998b; Saito *et al.*, 1993), whilst a more recent study has reported no change in the response to carbachol in M_2 compared to M_3 mediated bladder contractions (Stevens *et al.*, 2007). As muscarinic receptors have been shown to couple to different G proteins under different conditions, as previously discussed, it could be that the response of the M_2 receptor to stimulation by carbachol is also altered under varying conditions, therefore, the role of the M_2 receptor in the decreased responses seen in this present study cannot be accurately presumed.

In conclusion, the data obtained in this chapter supports the original hypothesis that the functional receptor subtype in the entire canine urinary bladder is the M_3 receptor, and that neutering a canine shifts the predominant functional muscarinic receptor subtype of the detrusor muscle from the M_3 to the M_2 receptor. The data also suggests a potential role of the M_2 receptor in mediating or facilitating contraction of the bladder in entire animals. It is hypothesised that the shift from M_3 to M_2 mediated bladder contraction in neutered canines might contribute to the decreased contractile response of detrusor strips, to carbachol, seen *in vitro* in this group, and as such may be involved in the development of acquired urinary incontinence. Further investigations involving a greater number of animals and an increased range of muscarinic receptor subtype specific antagonists is needed to confirm the role these receptor subtypes play in mediating contraction of the canine urinary bladder. Further investigation involving animals known to be suffering

from acquired urinary incontinence is likewise required to determine the role of these receptors in that disease state.

7 Effects of Acute Incubation with Reproductive Hormones on *In Vitro* Responses of Canine Detrusor Muscle Strips to Muscarinic and Electrical Stimulation

7.1 Introduction

Urinary incontinence is defined as the unconscious and involuntary loss of urine (Abrams *et al.*, 2002) and, in the canine, it is an increasingly recognised and presently incurable problem. Acquired urinary incontinence accounts for the majority of cases of urinary incontinence seen in the canine and is reported to affect up to 20% of all neutered bitches (Arnold *et al.*, 1989) but less than 1% of intact bitches (Holt *et al.*, 1993) and is rarely reported in male canines. It has been established that there is a direct relationship between acquired urinary incontinence in the canine and neutering (Thrusfield, 1985) and it has been proposed that acquired urinary incontinence occurs as a consequence of hormonal, vascular and/or neurological changes (Thom *et al.*, 1998) within the bladder rather than mechanical damage to the lower urinary tract as a direct result of surgery (Gregory, 1994).

Acquired urinary incontinence in the bitch was thought to be caused solely by a decrease in resting urethral pressure (Holt, 1988; Rosin *et al.*, 1981), but as this is not a defining characteristic of the condition (Holt, 1988) it is now thought that multiple causative factors are involved in predisposing animals to this condition. The results of previous *in vitro* studies (Chapter 4) suggest that functional changes occur within the detrusor muscle as a result of neutering in the canine. Specifically, regardless of gender, neutering leads to a decrease in the maximal contractile response of the detrusor muscle to both muscarinic and electrical field stimulation and to a decreased sensitivity to muscarinic stimulation. These results are similar to those reported for the detrusor muscle from postmenopausal women who suffer from urge urinary incontinence, relative to their premenopausal counterparts, and can occur either alone, as impaired contractility of the bladder, or in conjunction with idiopathic detrusor instability (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b).

Although there is insufficient data relating to the exact aetiology and pathophysiology of acquired urinary incontinence in neutered bitches and post menopausal women to allow determination of commonality of cause, it is intriguing that both groups are subject to a reduction in gonadal steroids and associated hormonal changes. This has led to the

hypothesis that the changes in steroid and associated hormones are mechanistically involved in the development of urinary incontinence in both humans (Stenberg *et al.*, 1995; Thom *et al.*, 1998) and canines (Arnold *et al.*, 1989; Thrusfield, 1985). Post menopause and neutering there is a significant decrease in circulating endogenous oestrogen and this has long been thought to be a causative factor in the development of urinary incontinence in both the bitch (Finco *et al.*, 1974) and woman (Freedman, 2002). Indeed, current recognised treatments for acquired urinary incontinence in the bitch include supplemental exogenous oestrogen which has been reported to restore continence, albeit temporarily, in up to 60% of animals (Mandigers *et al.*, 2001). Supplemental oestrogen has been reported to increase the resting tone of the urethra (Nickel, 1998) and it is possible that an increase in circulating oestrogen concentrations may also improve bladder function in terms of compliance, contractility and elasticity. Based on limited urodynamic studies reported in rats and canines (Fleischmann *et al.*, 2002; Nickel, 1998) all these factors could act to improve continence. *In vitro* measurements of contractility and responsiveness, however, have not been made in steroid treated tissues collected from animals suffering from acquired urinary incontinence.

In addition to the primary effects of ovarian oestrogen on the bladder, the decrease in oestrogen post neutering / menopause also interrupts the feedback mechanisms that act at the level of the hypothalamus and pituitary gland to regulate the secretion of additional hormones. The removal of oestrogen negative feedback leads to an increase in GnRH secretion and a concomitant increase in the secretion of both FSH and LH (Burger, 1996; Olson *et al.*, 1992; Reichler *et al.*, 2004). It has been hypothesized that this increase in gonadotrophins may be partially responsible for the development of urinary incontinence in both the canine (Reichler *et al.*, 2004) and woman (Tao *et al.*, 1998) and studies have demonstrated the presence of receptors for the gonadotrophins in the urinary bladder (Ponglowhapan *et al.*, 2007a; Reichler *et al.*, 2007; Tao *et al.*, 1998). Further support for an indirect effect of steroid removal on bladder function has been provided by a recent study in neutered canines suffering from acquired urinary incontinence which reported that administration of GnRH analogues, such as to decrease serum LH and FSH levels aided continence (Reichler *et al.*, 2006b). The study, however, did not address the mechanism through which these effects were mediated.

It could be hypothesised therefore that exposure to elevated concentrations of GnRH, LH and FSH may have a negative effect on bladder function and that treatment with exogenous hormones, to counteract post neutering changes in their secretion, could be beneficial with regard to urinary continence. In this study the hypothesis that acute exposure of the canine

detrusor to elevated concentrations of GnRH, LH, FSH or oestrogen will alter the contractility and sensitivity of strips of canine detrusor to muscarinic and electrical field stimulation was tested. More specifically, it was hypothesised that acute incubation with oestrogen would improve contractility in isolated strips of detrusor muscle from neutered canines and acute incubation with GnRH, LH and FSH would decrease contractility in isolated strips of detrusor muscle from entire canines.

7.2 Materials and Methods

7.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissue from a total of 20 canines was included in the study, although not all animals were included in each protocol. The study population had a mean age of 6.1 ± 0.6 years (range 2-14 years) and a mean weight of 23.6 ± 2.1kg (range 9-45kg). The canines were split into two groups depending on gonadal status with both male and female canines being used for the GnRH, FSH and LH studies, whilst only bitches were used for the oestrogen study. The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases, tissue was collected with full informed owner consent, within 2 hours of euthanasia (intravenous overdose of pentobarbitone) for reasons other than scientific investigation. The majority of animals had been euthanized for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. In all cases, a detailed history of each animal was taken and a gross post-mortem study of the entire urinary system was performed; any animals with a history of, or gross pathological signs of, urinary tract disease were excluded from the study.

7.2.2 Preparation of Tissue

For all tissue bath protocols, bladder tissue was used within 2 days of euthanasia of the animal (see chapter 3). To maximise the use of tissues a number of the animals included in this study were also included in the studies described elsewhere in this thesis.

Preparation of detrusor strips was as for chapter 4, with muscle strips being mounted on fixed hooks for carbachol concentration response protocols (7.2.4, 7.2.5) and in Ag-AgCl ring electrodes for electrical stimulation protocols (7.2.6, 7.2.7).

Before addition of the hormones, strips were tensioned until stable at 4g and were re-tensioned as required regularly throughout the incubation period. During incubation procedures, the isolated strips of detrusor muscle were maintained in the tissue baths at 37°C, were bathed in standard Krebs solution (chapter 3) with the addition of the hormone in question and were continually aerated with 95% O_2 / 5% CO_2.

7.2.3 Hormone Preparations

GnRH (luteinising hormone releasing hormone human acetate salt, Sigma-Aldrich, UK) was diluted in water (BDH, RNase and DNase free) to give a stock solution of 100mg/ml which was stored at -20°C. On the day of each experiment, the stock solution was diluted in Krebs solution and was added to each tissue bath as described below to give a final bath concentration of 10ng/ml. As there are no reports of concentrations of GnRH in the peripheral circulation of canines this concentration was chosen as it represents a concentration marginally higher than that found to produce maximal increases in FSH and LH production when administered acutely (hours) and to produce a decrease in FSH and LH production when administered chronically (days) (Concannon, 1993).

LH (porcine LH, supplied by A.F. Parlow of the National Hormone & Peptide Program, California, USA) was diluted in water (BDH, RNase and DNase free) to give a stock solution of 5mg/ml which was stored at -20°C. On the day of each experiment the stock solution was diluted in Krebs solution and was added to each tissue bath, as described below, to give a final bath concentration of 10ng/ml. This concentration was chosen as concentrations of LH in the peripheral circulation of a long-term (>42 week) neutered bitch average 8.3ng/ml (Reichler *et al.*, 2004).

FSH (canine FSH, Tucker Endocrine Research Institute LLC, USA) was diluted in water (BDH, RNase and DNase free) to give a stock solution of 1mg/ml which was stored at -20°C. On the day of each experiment, the stock solution was diluted in Krebs solution and was added to each tissue bath, as described below, to give a final bath concentration of 100ng/ml. This concentration was chosen as concentrations of FSH in the peripheral circulation of a long-term (>42 week) neutered bitch average 75ng/ml (Reichler *et al.*, 2004).

Oestrogen (Estradiol, standards from Estradiol MAIA calibrator 6, Bio-Stat, UK) was obtained in concentrations of 500pg/ml, 1500pg/ml and 5000pg/ml and were stored at 4°C. These were added to the tissue baths to give final bath concentrations of 3.3pg/ml, 15pg/ml and 50pg/ml. These concentrations were chosen as they spanned the range of oestrogen concentrations seen in canines during anoestrus (1-5pg/ml) and oestrus (40-60pg/ml) (Concannon, 1986a).

7.2.4 Carbachol Concentration Response Protocol with GnRH, LH or FSH

All strips (≥ 8 per animal) underwent a full carbachol concentration response protocol (4.2.4), the full results of which are incorporated into the data presented in chapter 4.

Following completion of the initial carbachol concentration response protocol, the muscle strips were washed repeatedly, allowed to rest for 30 minutes and the tension adjusted until stable at 4g resting tension (Chapter 3). GnRH, LH or FSH were then added to two baths each, to give the bath concentrations described above, leaving two of the eight strips without any additional hormone, as time controls. Strips were incubated for 2 hours, after which time they were re-tensioned to 4g initial tension (as necessary) before a further full carbachol concentration response protocol was carried out.

7.2.5 Carbachol Concentration Response Protocol with Oestrogen

For this protocol only female animals were used and were split into two groups depending on gonadal status; entire and neutered. All strips (~ 8 per animal) underwent a full carbachol concentration response protocol (4.2.4), the results of which are incorporated into the data presented in chapter 4.

After the initial concentration response protocol was carried out all strips were washed repeatedly, allowed to rest for 30 minutes and then tensioned until stable at 4g resting tension. Oestrogen, at each of the three concentrations described above, was then added to two baths each, leaving two of the eight strips without any additional hormone, as time controls. Strips were incubated with oestradiol for 2 hours, after which time the strips were re-tensioned to 4g initial tension, as necessary, before a further full carbachol concentration response protocol was carried out.

The strips were then washed repeatedly, as before, rested for 30 minutes and tensioned until stable at 4g initial tension before re-addition of oestrogen, at the concentrations to which the strips were previously exposed and incubated with this concentration of oestradiol for a further 22 hours. After the incubation, the strips were again re-tensioned to 4g initial tension and a further full carbachol concentration response protocol carried out.

7.2.6 Neurogenic Electrical Field Stimulation Protocol with GnRH, LH or FSH

All strips (≥8 per animal) underwent a neurogenic electrical field stimulation protocol (4.2.5), the results of which are incorporated into the data presented in chapter 4.

After the initial frequency response protocol was carried out all strips were washed repeatedly, allowed to rest for 30 minutes and then tensioned until stable at 4g resting tension. GnRH, LH or FSH were then added, to two baths each, to give the bath concentrations described above, leaving two of the eight strips without any additional hormone to be the time controls. Strips were incubated for 2 hours, after which time the strips were re-tensioned to 4g initial tension, as necessary, before a full frequency response protocol was carried out.

7.2.7 Neurogenic Electrical Field Stimulation Protocol with Oestrogen

All strips (≥ 8 per animal) underwent a neurogenic electrical field stimulation protocol (4.2.5), the results of which are incorporated into the data presented in chapter 4.

After the initial frequency response protocol was carried out, all strips were washed repeatedly, allowed to rest for 30 minutes and then tensioned until stable at 4g resting tension. Oestrogen, at each of the three concentrations described above, was then added to two baths each, leaving two of the eight strips without any additional hormone to be the time controls. Strips were incubated with oestrogen for 2 hours, after which time the strips were re-tensioned to 4g initial tension, as necessary, before a full frequency response protocol was carried out.

The strips were then washed repeatedly as before, rested for 30 minutes and tensioned until stable at 4g initial tension before oestrogen was reapplied, at the concentration to which the strips were exposed previously, and the strips incubated for a further 22 hours after which time the above procedure was repeated.

7.2.8 Data Analysis

All data were normalised for wet weight of tissue and are expressed as g/mg of wet tissue with results presented as mean ± s.e.mean (n = number of animals).

A minimum of 2 strips of bladder tissue per animal were analysed for each hormone, at each incubation time and the mean results from these strips used in further calculations where applicable. Data were graphed and statistical analysis performed using GraphPad Prism® v.5 software. In all cases the maximal tension was defined as the mean maximal tension of all the strips from that animal for that protocol. Comparisons within (time control) and between (differences in responses following hormone incubations) response protocols were made using analysis of variance (ANOVA) with Bonferroni as a post-test. A probability (P) less than or equal to 0.05 was considered significant. Correlations between responses and the age and weight of the animals were conducted using Spearman's test with a significance threshold for P (two tailed) < 0.05.

Statistical analysis of the responses seen in the detrusor strips used as time controls indicated that there was no significant effect of time on either the maximal response or sensitivity of the strips to carbachol when repeated concentration response curves were run as required to test for effects of hormone incubation (Figs. 7-1 and 7-2). There was also no effect of time on the response of the tissue strips to neurogenic field stimulation (Figs. 7-3 and 7-4). Therefore, the effects of hormone treatment on the responses of the muscle strips were statistically compared by analysis of variance.

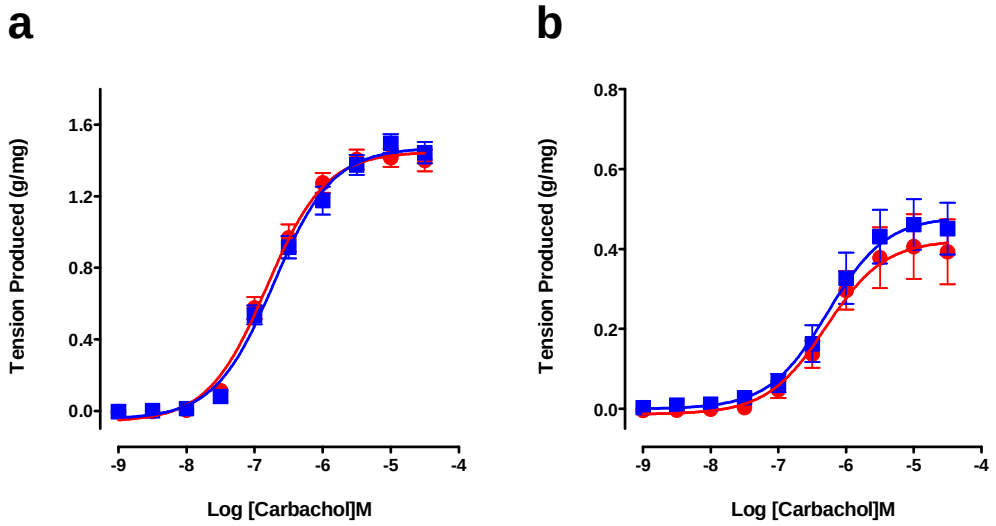

Figure 7-1. Time control cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle for GnRH, LH and FSH studies. ● Time 0 hours, ■ Time 2.5 hours. Each point is the mean ± s.e.mean of observations from n animals. a, entire animals, n=8; b, neutered animals, n=7.

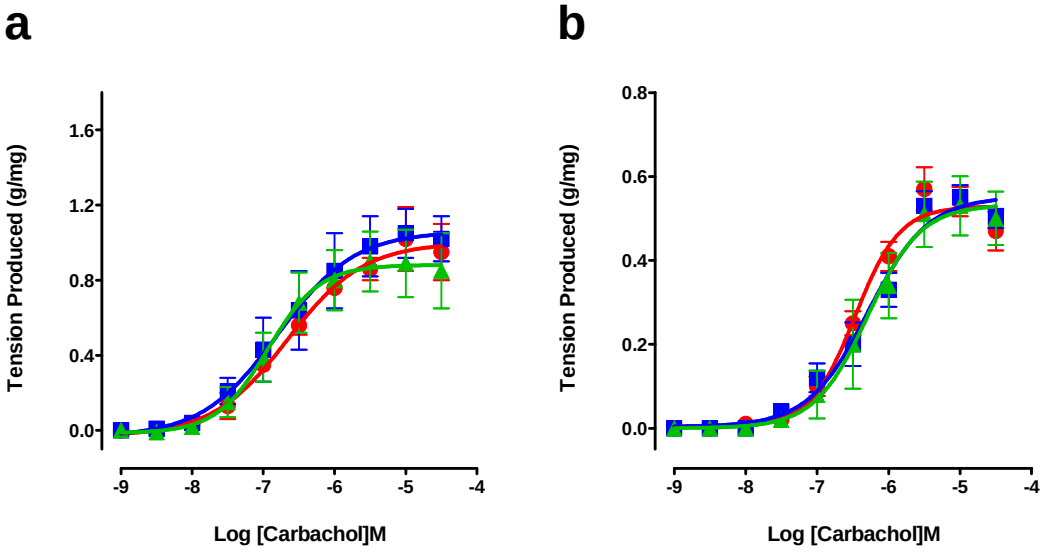

Figure 7-2. Time control cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips for oestrogen studies. ● Time 0 hours, ■ Time 2.5 hours, ▲Time 24 hours. Each point is the mean ± s.e.mean of observations from n animals. a, entire bitches, n=3; b, neutered bitches, n=3.

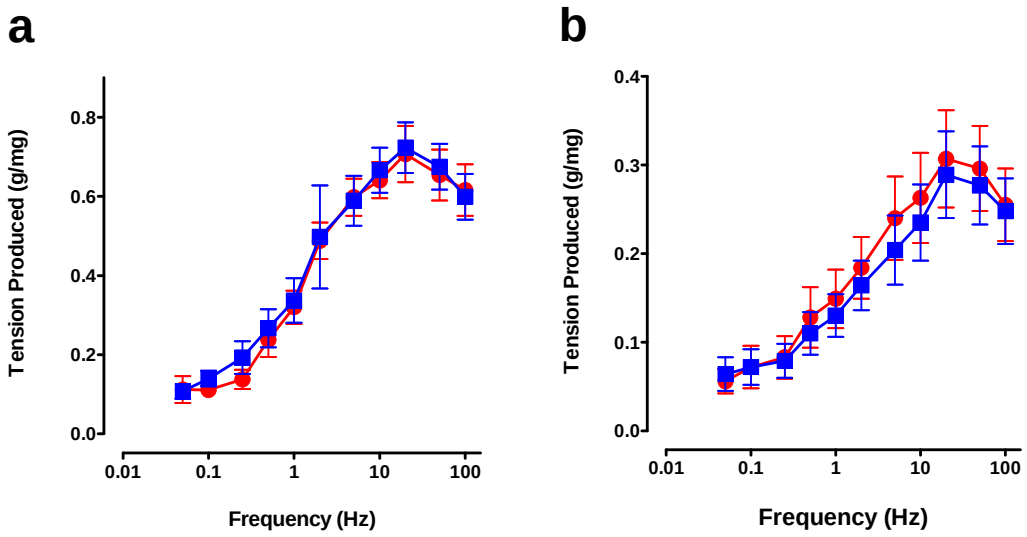

Figure 7-3. Time control frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips for GnRH, LH and FSH studies. ● Time 0 hours, ■ Time 2.5 hours. Each point is the mean ± s.e.mean of observations from n animals. a, entire animals, n=7; b, neutered animals, n=6.

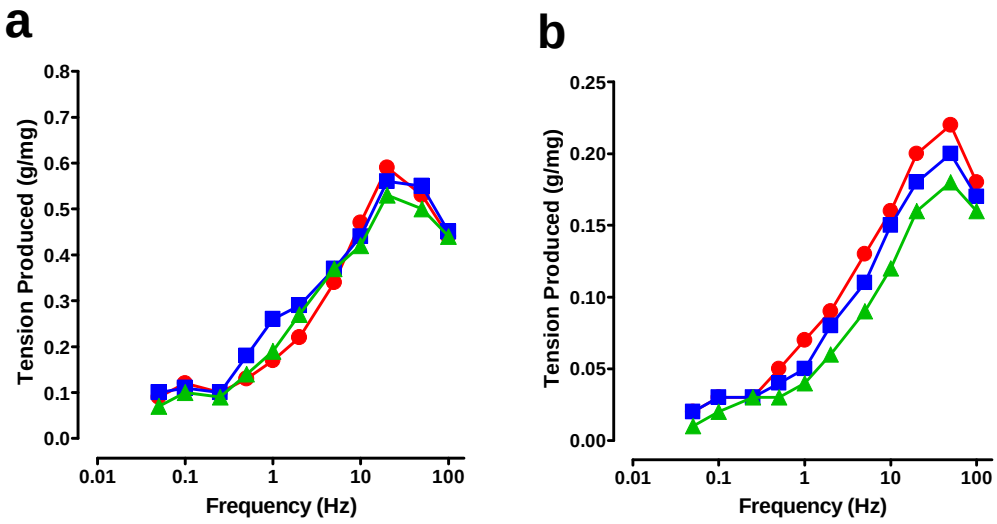

Figure 7-4. Time control frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips for oestrogen studies. ● Time 0 hours, ■ Time 2.5 hours, ▲ Time 24 hours. Each point is the mean of observations from n animals. a, entire animals, n=2; b, neutered animals, n=2.

7.3 Results

7.3.1 Acute Effects of Incubation with GnRH, LH or FSH on Responses to Carbachol Stimulation

In all groups, when exposed to the first dose response protocol, dose dependant contractions were observed in response to carbachol. As presented in chapter 4, neutering, regardless of gender, was associated with a significant decrease in the maximum contractile response and sensitivity of the isolated strips of bladder smooth muscle to carbachol, relative to tissue from entire animals ($p<0.05$). As no significant differences were seen between genders, the results of the males and females within each gonadal status group were combined for all further analyses in this study.

No statistically significant effects of incubation with GnRH, LH or FSH (for 2 hours), were seen on either maximal response or sensitivity to carbachol, of the isolated strips of bladder smooth muscle in animals of either gonadal status (Fig. 7-5). In addition, statistical analysis indicated that there was no statistically significant effect of the age or weight of the animal on the response to carbachol following hormone incubation.

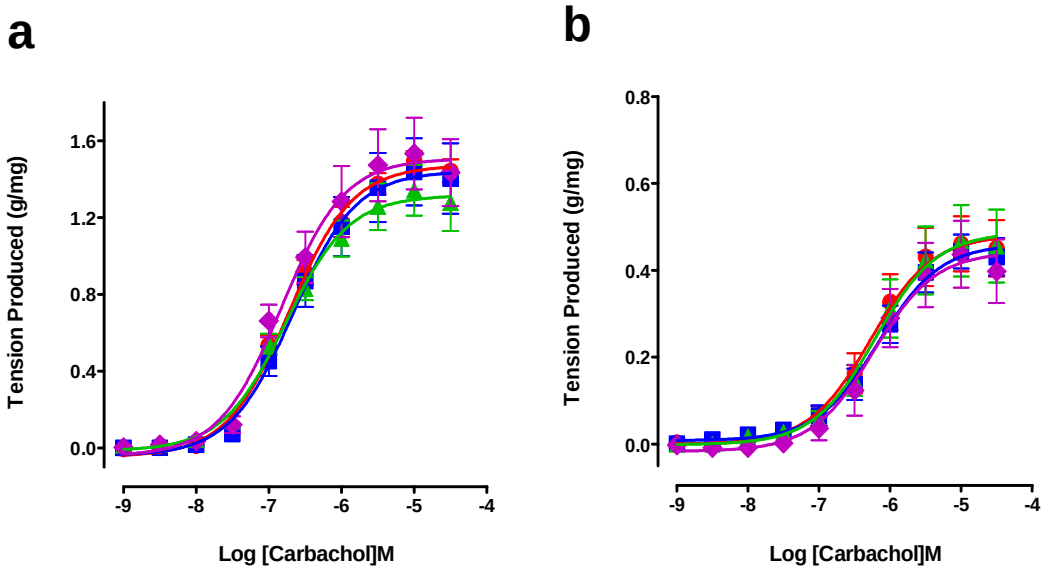

Figure 7-5. Cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips following a 2 hour incubation: ● Control, ■ GnRH (10ng/ml), ▲ LH (5.0µg/ml), ♦ FSH (100ng/ml). Each point is the mean ± s.e.mean of observations from n animals. a, entire animals, n=8; b, neutered animals, n=7.

7.3.2 Effects of Acute Exposure to Elevated GnRH, LH or FSH on Responses to Neurogenic Field Stimulation

In all groups, during both stimulation protocols, frequency dependant contractions were observed in response to electrical field stimulation. The effects of neutering on the response of isolated strips of bladder smooth muscle to neurogenic electrical field stimulation were similar to those of carbachol, with a significant decrease ($p<0.05$) in the maximal contractile response in neutered compared to entire animals, as presented and discussed in chapter 4. As with the results obtained for the carbachol dose response study, described above, there was no gender difference in the responses to neurogenic electrical field stimulation, therefore, the results obtained for animals of both sexes were combined to provide 'entire' and 'neutered' groups for this study.

Statistical analysis revealed no significant acute effect of incubation, for 2 hours, with GnRH, LH or FSH on the maximal response of the isolated strips of bladder smooth muscle to neurogenic field stimulation in either group (Fig. 7-6). As with carbachol, there was no statistically significant effect of the age or weight of the animal on the response to neurogenic field stimulation.

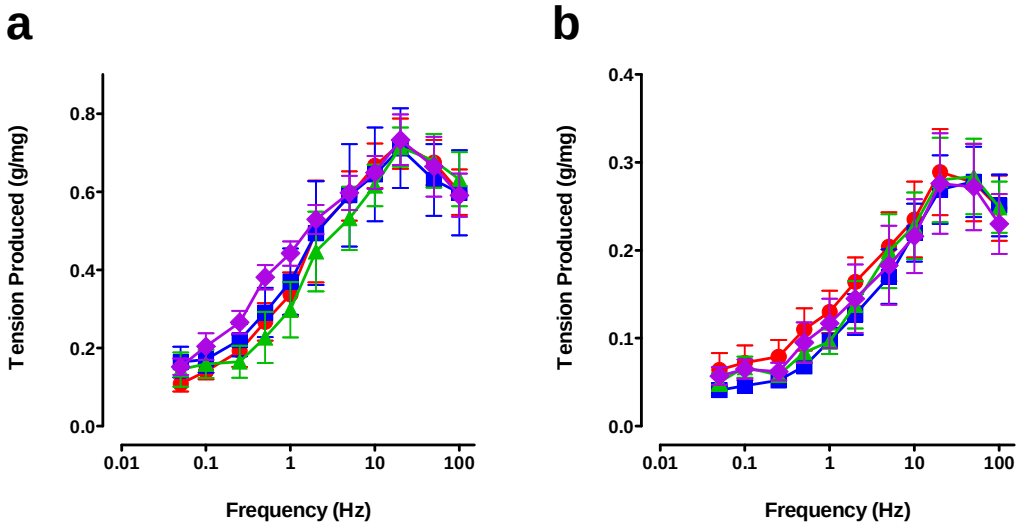

Figure 7-6. Frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips following 2 hour incubation with hormones: ● Control, ■ GnRH (10ng/ml), ▲LH (5.0µg/ml), ♦ FSH (100ng/ml). Each point is the mean ± s.e.mean of observations from n animals. a, entire animals, n=7; b, neutered animals, n=6.

7.3.3 Effects of Oestrogen on Detrusor Muscle Responses to Carbachol Stimulation

As presented and discussed in chapter 4, there was dose dependant contraction of the isolated strips of bladder smooth muscle when stimulated with carbachol, with strips from neutered bitches responding with significantly less sensitivity and maximal contraction than those from entire bitches. As oestrogen is primarily a hormone of the female reproductive cycle and as its absence is thought to play a role in the development of female acquired urinary incontinence only female animals were used for this section of the study.

There was no statistically significant effect of incubation with oestrogen for either 2 hours (Fig. 7-7) or 24 hours (Fig. 7-8), on the maximal response or sensitivity of the isolated strips of bladder smooth muscle to carbachol, in either gonadectomised or entire animals. In addition the statistical analysis indicated that there was no statistically significant effect of the age or weight of the animal on the response to carbachol.

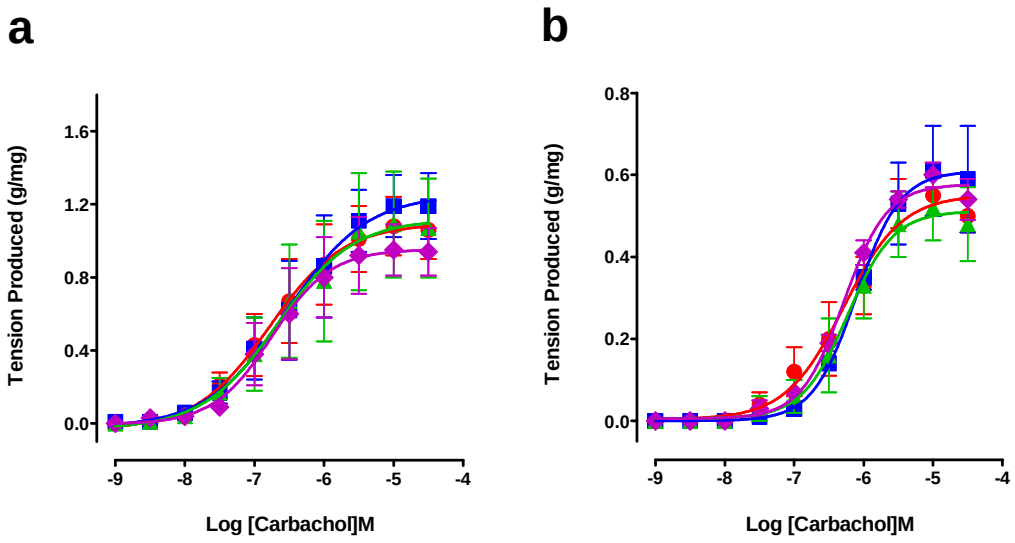

Figure 7-7. Cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips following a 2 hour incubation with varying concentrations of oestrogen: ● Control, ■ 3.3pg/ml, ▲ 15pg/ml, ♦ 33pg/ml. Each point is the mean ± s.e.mean of observations from n animals. a, entire bitches, n=3; b, neutered bitches, n=3.

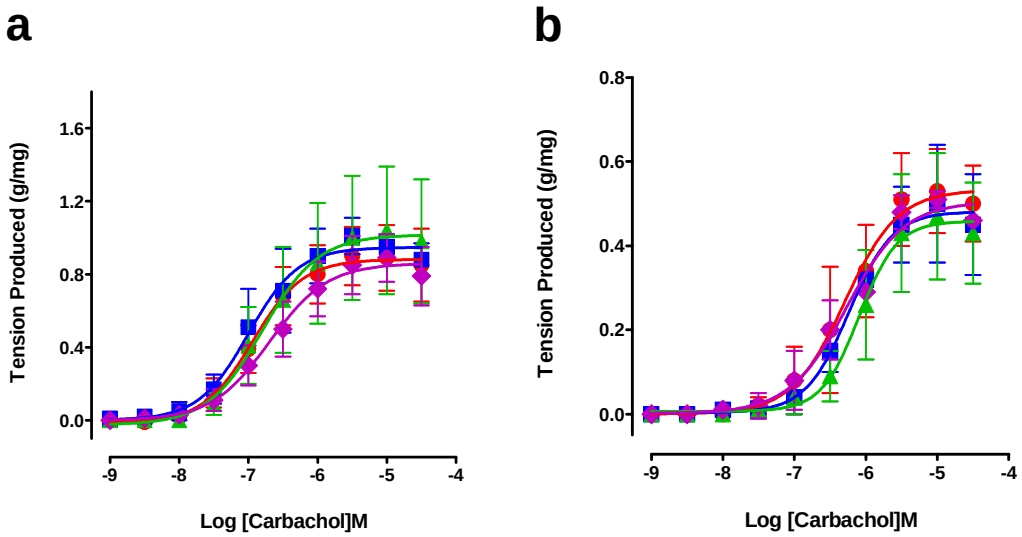

Figure 7-8. Cumulative concentration response curves to carbachol in isolated canine urinary bladder smooth muscle strips following a 24 hour incubation with varying concentrations of oestrogen: ● Control, ■ 3.3pg/ml, ▲ 15pg/ml, ♦ 33pg/ml. Each point is the mean ± s.e.mean of observations from n animals. a, entire bitches, n=3; b, neutered bitches, n=3.

7.3.4 Effects of Oestrogen on Detrusor Muscle Responses to Neurogenic Field Stimulation

In all groups, frequency dependant contractions were observed in response to electrical field stimulation as previously described. As with carbachol, above, only bitches were included in this study and the maximal contractile response of the neutered bitches was significantly lower than that of the entire bitches. Unfortunately, due to equipment failure, data was only available for 2 animals from each group. There was no effect of either 2 (Fig. 7-9) or 24 hour incubation (Fig. 7-10) with oestrogen on the response of the isolated strips of bladder smooth muscle to neurogenic field stimulation in animals of either group.

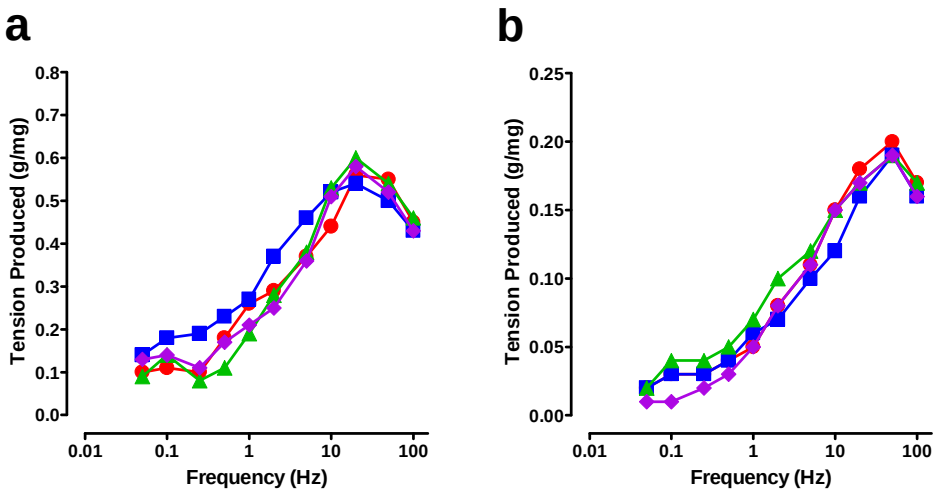

Figure 7-9. Frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips following a 2 hour incubation with varying concentrations of oestrogen: ● Control, ■ 3.3pg/ml, ▲ 15pg/ml, ♦ 33pg/ml. Each point is the mean of observations from n animals. a, entire animals, n=2; b, neutered animals, n=2.

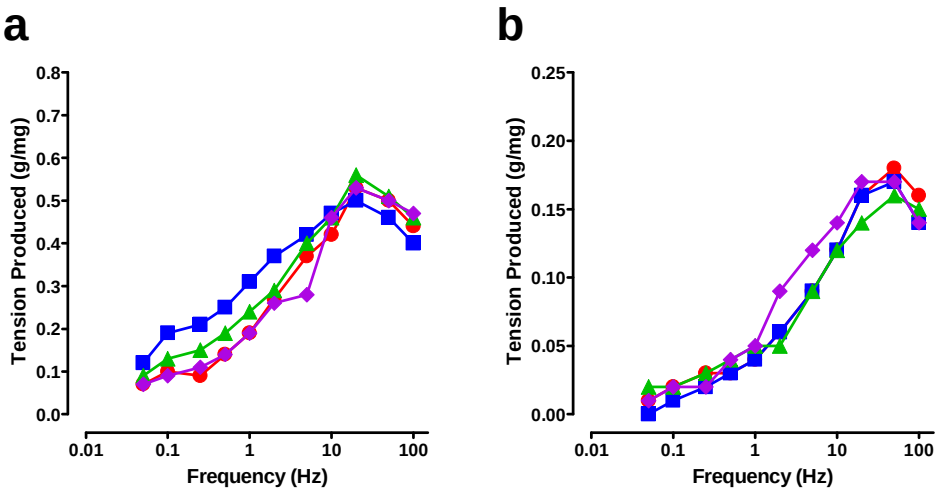

Figure 7-10. Frequency response curves to neurogenic field stimulation in isolated canine urinary bladder smooth muscle strips following a 24 hour incubation with varying concentrations of oestrogen: ● Control, ■ 3.3pg/ml, ▲ 15pg/ml, ♦ 33pg/ml. Each point is the mean of observations from n animals. a, entire animals, n=2; b, neutered animals, n=2.

7.4 Discussion and Conclusion

Based upon the reported effects of GnRH analogues and oestradiol in the treatment of acquired urinary incontinence and the observed differences in responsiveness of the detrusor in gonadectomised animals in chapter 4 of this thesis, this study set out to test the hypothesis that acute exposure to GnRH, LH, FSH and oestrogen may alter responsiveness and sensitivity of isolated strips of detrusor muscle to muscarinic and electrical field stimulation. From the results obtained it is possible to see that under the study conditions used this hypothesis was refuted.

This thesis has previously shown that neutering a canine, of either gender, causes a decrease in sensitivity and contractility to both muscarinic and neurogenic field stimulation (chapter 4). The observed decrease in sensitivity and contractility is similar to that described in a proportion of post-menopausal women where it is thought to play an important role in the development of urinary incontinence (Mills *et al.*, 2000) and it was postulated that the changes in bladder contractility and sensitivity seen in neutered canines may play a similar role in the development of acquired urinary incontinence in the bitch. Although there are a number of theories as to why the known changes in sensitivity and contractility of the detrusor muscle occur, the longest held and most popular theory is that changes in reproductive hormone levels post neutering or post-menopause are causative factors (Arnold *et al.*, 1989; Burger, 1996; Thrusfield, 1985; Zhu *et al.*, 2001). *In vivo* treatment of animals suffering from acquired urinary incontinence with either supplemental oestrogen or GnRH analogues to decrease LH and FSH concentrations has been shown to improve continence in a number of animals (Angioletti *et al.*, 2004; Janszen *et al.*, 1997; Reichler *et al.*, 2003). It is, therefore, plausible that changes to these hormone concentrations may cause changes in sensitivity or contractility of the detrusor muscle to stimulation via the muscarinic pathway, the primary pathway responsible for bladder emptying (Chess-Williams, 2002), possibly by altering muscarinic receptor expression as has been demonstrated in the rabbit in response to oestrogen (Batra *et al.*, 1989).

While the results of this study do not support the original hypothesis, consideration must be given to the timescale of the post-neutering changes in both hormone concentrations and continence, relative to the hypothesis and study design. This study specifically investigated the effects of acute (2 and 24hr) exposure to altered hormone concentrations, however, while there are acute hormonal changes after neutering, peripheral hormone concentrations continue to change over a number of months (Reichler *et al.*, 2004) and the

development of acquired urinary incontinence can take months or years to manifest. Furthermore, the responses of bitches suffering from acquired urinary incontinence to hormonal treatment can take weeks to fully develop (Angioletti *et al.*, 2004; Reichler *et al.*, 2003; Trigg *et al.*, 2001) which would suggest that chronic changes in reproductive hormones may be necessary to effect a change in response of the tissues. Due to experimental and ethical constraints tissue was only obtained from animals which had no history of prior drug or hormonal treatment, therefore, the exposure of the bladder tissue to supplemental hormones could only occur *in vitro* and thus only relatively acute effects of hormone exposure were investigated. It is possible that this relatively short incubation with hormones was insufficient to evoke any changes in sensitivity and contractility that may have been seen in response to chronic hormonal changes. Furthermore, changes in responsiveness may be secondary to alterations in bladder structure or receptor number brought about by chronic hormone exposure thus requiring a prolonged time-course before functional changes are evident *in vitro*. This later hypothesis is supported by reports that chronic changes in oestrogen concentrations over a number of weeks and months can cause structural changes to the urinary bladder in rodents (Fleischmann *et al.*, 2002) and that chronic changes in gonadotrophin concentrations post-menopause in women can cause changes in receptor numbers within the bladder (Tao *et al.*, 1998).

To test the long term influence of hormonal changes on *in vitro* bladder responsiveness in the canine would require the use of animals that had been subjected to known changes in hormone concentrations over a chronic time frame. For this, age-matched, breed-matched animals, ideally littermates, would be needed to allow some animals to be treated with supplemental hormones over a set number of weeks and months, whilst some animals were used as time controls. Once treatment over the predestined time period was over, the animals would be sacrificed and isolated strips of detrusor muscle from each animal subjected to a full carbachol and neurogenic field stimulation protocol as described in 7.2.4 - 7.2.7 above. This would allow direct comparisons of *in vitro* bladder function in chronically hormonally treated animals and in matched control animals, however, this was not feasible within the confines of this project.

In conclusion, therefore, the results of this study demonstrate that acute incubation with oestrogen, GnRH, FSH or LH has no effect on the *in vitro* responses of isolated strips of canine detrusor muscle to muscarinic and neurogenic field stimulation. This disproves the original hypothesis which stated that alterations in responsiveness of bladder strips to the acute addition of these hormones would occur. However, these results do not allow a judgement to be made as to whether chronic exposure to altered concentrations of

hormones would affect the responsiveness of the detrusor muscle to muscarinic or neurogenic field stimulation, by either causing alterations in the structure of the urinary bladder, or the number and distribution of receptors within it. Further investigation into possible causes of the decrease in sensitivity and responsiveness of strips of detrusor muscle as described in chapter 4 is therefore warranted.

8 mRNA Expression of M_1, M_2 and M_3 Receptors in the Canine Urinary Bladder – Effects of Neutering

8.1 Introduction

The propensity to develop acquired urinary incontinence in the bitch, a debilitating and currently incurable condition, has long been linked to neutering (Arnold *et al.*, 1989; Holt *et al.*, 1993; Thrusfield, 1985). Despite this positive correlation, the exact aetiology and pathophysiology of the condition in the neutered bitch remains undetermined. It is understood that there is a decrease in urethral closure pressure following neutering in the bitch (Holt, 1988) and this has been considered a primary causative factor for the development of acquired urinary incontinence for a number of years. It has also been shown, however, that raising the urethral closure pressure does not result in continence in all canines (Barth *et al.*, 2005), which has lead to the hypothesis that further factors are involved in the development of this condition. The results of chapter 4 (Coit *et al.*, 2008), demonstrate that the detrusor muscle of gonadectomised canines has a lower, *in vitro,* sensitivity and contractility to muscarinic and neurogenic field stimulation than that of gonad intact animals and this is hypothesised to contribute to the development of acquired urinary incontinence.

The basis for the functional change in bladder contractility *in vitro* is not yet fully understood, however, a number of factors have been hypothesised to be involved including a change in bladder structure and a change in the number and subtype of muscarinic receptors present on and within the bladder wall. To help address this chapter 5 investigated whether neutering was associated with changes in bladder structure in terms of changes in percentage collagen. The results demonstrated that, in the canine, neutering a bitch results in structural changes in the bladder wall, namely an increase in the percentage of collagen present within the detrusor, but interestingly that this change is not seen in the neutered male (chapter 5). As neutering is associated with changes in bladder contractility and sensitivity in both male and female canines these results suggest that changes in bladder structure alone cannot account for the observed changes in bladder function and thus that an additional, separate mechanism must also be involved.

It is known that the muscarinic receptor effector pathway is the primary pathway responsible for bladder contraction and emptying, and that the M_3 receptor is the principal receptor subtype involved in normal urination (Chess-Williams, 2002; Chess-Williams *et al.*, 2001; Fetscher *et al.*, 2002). It is also known that the M_1 and M_2 receptor subtypes may have a minor role to play in bladder contraction in healthy individuals (Frazier *et al.*, 2007). Studies in both rats and humans have shown that the receptor complement of the bladder and the subtypes involved in normal bladder contraction can change with different disease states (Andersson *et al.*, 2004b); for example, following denervation of the bladder the M_2 receptor subtype becomes the predominant receptor subtype responsible for bladder contraction (Braverman *et al.*, 1998b; Pontari *et al.*, 2004). In addition, chapter 6 of this thesis demonstrated that although the M_3 receptor was responsible for detrusor muscle contraction in entire canines *in vitro*, it was the M_2 receptor that was responsible for detrusor contraction in neutered canines. Furthermore, a limited study in pigs has suggested that bladder contractions mediated via stimulation of the M_2 receptor may be weaker than those mediated by the M_3 receptor, and that the sensitivity of the M_2 receptor to carbachol may be less than that of the M_3 receptor (Yamanishi *et al.*, 2000). Together these results would suggest that a change in subtype, number or the ratio of muscarinic receptors may result in altered detrusor function and could therefore provide a means of explaining the results described in chapter 4 of this thesis.

This study will therefore test the hypothesis that there is a difference in the expression of mRNA for the M_1, M_2 and M_3 receptor subtypes within the urinary bladder of neutered relative to gonadally intact canines. If this hypothesis holds true then these differences may be involved in the decreased sensitivity and contractility of the urinary bladders of the neutered animals to muscarinic stimulation *in vitro* seen in chapter 4, and may be involved in the development of acquired urinary incontinence.

8.2 Materials and Methods

8.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissue from a total of 75 canines was included in the study. The study population had a mean age of 5.6 ± 0.7years (range 1-16 years) and a mean weight of 22.6 ± 1.5kg (range 8-50kg). The canines were split into five groups depending on gender, gonadal status and incidence of acquired urinary incontinence: entire and neutered males, entire and neutered female, plus neutered females known to be suffering from acquired urinary incontinence. The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases, tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbatone) with full informed owner consent, for reasons other than scientific investigation. The majority of animals had been euthanized for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. A full gross post mortem of the urinary tract was performed before tissue harvesting and any animals with a history or signs of gross pathological urinary tract disease other than acquired urinary incontinence (e.g. tumours, cystitis) were excluded from the study.

To maximise use of tissue and enable comparisons between different parameters, a number of the animals included in this study were also included in the studies described elsewhere in this thesis.

8.2.2 Preparation of Tissue

Full thickness biopsies (2cm by 2cm) were collected from the area of the bladder dome. Samples were immediately frozen in liquid nitrogen and stored at -80°C until required for mRNA studies.

8.2.3 mRNA Extraction

RNA was extracted from small samples (~1mg) of frozen tissue by addition of an appropriate volume of ice cold TRIzol® Reagent (Invitrogen, Paisley, UK), usually 1.0ml, in a 2ml LysingMatrixD tube (Q Biogene) and homogenisation using a Ribolyser (Hybaid, UK) for five 30-second periods or as required for complete disruption of tissue architecture. To isolate and purify the mRNA one tenth volume of chloroform was added to each sample and the contents mixed by vortexing. Samples were then put on ice for 4 minutes before being centrifuged for 15 minutes at 12,500rpm. The upper aqueous layer was removed, the volume noted, and transferred to a 1.5ml eppendorf tube. An equal sample volume of Isopropanol (Sigma, UK) was added to each sample and the tube placed on ice for 30 minutes to allow precipitation of the mRNA before centrifugation for 15 minutes at 12,500rpm. The centrifugation formed a pellet of the RNA precipitate. The supernatant was decanted from each sample and the pellets washed with 700µl 80% ethanol before further centrifugation for 8 minutes at 8,000rpm. The ethanol was then decanted and the pellets dried before resuspension of the mRNA in 30µl water (BDH, Rnase / Dnase free), 3µl of 3M NaAc (pH 7) and 60µl of absolute ethanol. Samples were then incubated for 30 minutes at -70ºC, before centrifugation for 15 minutes at 2500rpm. The supernatant was again decanted and the samples dried before being dissolved in 30µl of water (BDH, Rnase / Dnase free). The samples were then placed in a water bath at 65ºC for 5 minutes to ensure the RNA was in solution before being quick-frozen to -80ºC for storage.

8.2.4 Reverse Transcription

Master mixes were used in this protocol to allow more accurate pipetting of small volumes of reagents. They were made fresh each day and exact volumes were calculated depending on the number of samples to be reverse transcribed.

Reverse transcription was carried out by the addition of the equivalent of 7.33µl dH_2O (UV treated), 0.25µl dNTPs (ABgene, UK) and 0.13µl random hexamers (Invitrogen, UK), (all made up as a master mix), and 2.00µl of RNA template (protocol 8.2.3), for each sample, to a 0.5ml eppendorf. This was heated at 65ºC for 5 minutes in a water bath, then immediately chilled on ice. To this were added the equivalent of 1.00µl X5 RT buffer, 1.00µl DTT, 0.15µl RNAse inhibitor (RNAsin, Promega, UK) and 0.15µl M-MLV RT

(Invitrogen, UK) (all made up as a master mix). The mixture was then incubated in a water bath at 37ºC for 50 minutes, before the enzyme was inactivated by incubating at 70ºC for 15 minutes. The reaction product was then stored at -20ºC until required.

8.2.5 Polymerase Chain Reaction

The polymerase chain reaction allows amplification of specific target sequences of DNA using oligonucleotide primers, each complimentary to one end of the DNA target sequence. These primers are extended in a 3' direction by a thermo-stable DNA polymerase in a three-step reaction involving a high temperature denaturising step (95˚C), a low temperature-annealing step (50-55˚C) and an extension step (72˚C).

Primers were designed with reference to information obtained from the canine genome and equivalent sequences in other species held within GenBank. Primers were designed to be specific to the gene of interest and this specificity was verified by comparison with the entire GenBank database using BLAST. Potential primers were checked to ensure they did not contain sequence that would give rise to significant secondary and complimentary structures and then appropriate primers ordered from MWG Biotech, UK. Primers used in this study were between 17-28 bases long, with 50-60% GC content.

PCRs were conducted using an Ampitaq Gold Kit (Applied Biosystems, UK). Briefly, for each PCR reaction 3µl 10x buffer, 2.5µl $MgCl_2$ (2mM), 0.16µl taq polymerase, 0.3µl dNTPs, primers (0.3µl each, 20pmol) and 2µl cDNA template (protocol 8.2.4), made up with 21.5µl dH_2O (UV treated), were mixed on ice in 0.2ml domed topped eppendorf tubes. Samples were then loaded into a PCR express machine (Thermo Hybaid, UK) and run with the following conditions: initial denaturation at 95˚C for 3 minutes, then 35 cycles of 95˚C denaturation for 30 seconds, a primer-specific annealing temperature for 30 seconds and then 1 minute of 72˚C extension. A terminal extension step of 5 minutes at 72˚C was used. Non-template controls were included in all reactions to confirm that no contamination had been introduced that could give rise to false results.

8.2.6 Gel Electrophoresis

This allows the separation of DNA or RNA products on the basis of size and electric charge. The DNA molecule is negatively charged and will migrate through a gel matrix towards a positive electrode. Product size can be electrophoretically resolved by comparison with DNA size standards. Loading buffer, containing glycerol and bromophenol blue, was added to the PCR products to allow estimation of sample migration. Agarose gel matrix was used in this study. Agarose gels were 1% unless otherwise stated, they were prepared in 0.5x TBE buffer (0.045M Tris-borate + 0.001M EDTA, pH 8.0) and heated to induce dissolution of the agarose. While cooling, ethidium bromide was added (1µl ethidium to 100ml agarose gel) to permit visualisation of the DNA product under UV light. A voltage difference of 10V/cm was applied to the gel and it was run for as long as required to induce separation of the DNA fragments.

8.2.7 Purification of DNA from Agarose Gel

DNA fragments of interest i.e. bands of the estimated size, were extracted from gels and cleaned using the GenElute Gel Extraction Kit (Sigma, UK). The DNA fragment of interest was visualised under UV light, excised from the agarose gel, the gel block weighed and placed in a 1.5ml eppendorf. 3 gel volumes of Gel Solubilisation Solution were added to each gel block and the tubes incubated at 55ºC for 10 minutes before vortexing briefly to ensure complete solubilisation of the gel. A GenElute Binding Column G, one for each sample being extracted, was then placed in a 2ml collection tube and 500µl of the Column Preparation Solution added before centrifugation at 12,000rpm for 1 minute. The flow-through liquid was discarded. One gel volume of 100% Isopropanol was added to the tube containing the dissolved gel and the contents mixed until homogenous, before loading into the prepared binding columns. If the volume of the gel mixture was >700µl the sample was loaded into the column in 700µl portions. The columns were centrifuged at 12,000rpm for 1 minute after each loading and the flow-through liquid discarded. The columns were then washed by the addition of 700µl of Wash Solution and centrifuged at 14,000rpm for 1 minute. The flow-through liquid was again discarded and the tube centrifuged at 12,000rpm for a further 1 minute. Once washed the binding columns were transferred to fresh collection tubes and 50µl of Elution Solution added to the centre of the membrane before incubation at 65ºC for 1 minute and centrifugation at 12,000rpm for 1

minute. The columns were then discarded and the purified products kept on ice until required.

8.2.8 DNA Sequencing

Sequencing of PCR products was carried out with the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, UK). Amplification reactions were performed in a 0.2ml eppendorf containing 3µl Big Dye Buffer, 1µl Big Dye, 0.5µl sequence specific primer (3.2pmol concentration, sourced from MWG Biotech, UK) and 2µl of purified PCR product as template. Thermal cycling was performed at 96ºC for 30 seconds, 50ºC for 15 seconds and then 55ºC for 4 minutes for 25 cycles using a PCR Express Machine (Thermo Hybaid, UK).

The amplified product was purified by ethanol precipitation. To the reaction solution was added 8µl water and 32µl 95% ethanol to give a final concentration of ethanol of 60% ± 3%. This was left to stand at room temperature for 15-30 minutes to allow precipitation of extension products before centrifugation for 30 minutes at 14,000rpm at 4ºC. The supernatant was then removed and the pellet washed in 150µl 70% ethanol. Following further centrifugation at 14,000rpm at 4ºC for 10 minutes, the supernatant was removed, the pellet dried and re-suspended in 5µl formamide.

Sequencing was performed on the ABI Prism 3100 Genetic Analyser (ABI Biosystems, USA). The sequences were viewed and text files created using Chromas 2.3 (Technelysium Pty Ltd, AUS).

8.2.9 Real Time PCR

To quantify the differences in receptor mRNA expression between bladder samples, real-time PCR was used to measure relative mRNA levels in each sample. For all samples a standard housekeeping gene, actin, which is expressed in all eukaryotic cells, was used as a standard to allow the relative quantification of gene expression. For all experiments a non-template control (NTC) was included to confirm that no contamination had occurred

between samples or been included on the plate. If any contamination had been detected the results of that plate would have been discarded.

The primer and probe sequences used for the analysis were designed using Primer Express (Applied Biosystems, UK) with sequence information supplied from Genbank and our previous sequencing studies. All real time PCR primers and probes were ordered from Eurogentec (Southampton, UK).

Real time PCRs were carried out in a 25µl reaction volume in a 96 well plate format. The reaction was performed using the Amphitaq Gold kit (Applied Biosystems, UK). Each sample was run in duplicate therefore the reaction was performed in 31.16µl H_2O (DBH, Rnase / Dnase free), 6µl buffer, 6µl $MgCl_2$ (25mM), 0.6µl dNTPs and 0.25µl Taq (made up as a master mix). To this was added 2µl of probe and 1.5µl of each primer (forward and reverse). This was then split and placed into two adjacent wells on the plate. The thermal cycling consisted of an initial 7-minute 95ºC denaturation step then 35 cycles of 95ºC for 30 seconds denaturation with a 55ºC annealing step for 30 seconds, followed by an extension step for 1 minute at 72ºC. Thermal cycling and fluorescence detection was performed on a Stratagene Mx3000P machine (Agilent Technologies UK Limited, Cheshire, UK).

8.2.10 Statistical Analysis

Results for mRNA expression levels were analysed against both age and weight using multivariant analysis with $P < 0.05$ considered significant.

Results are given as mRNA expression levels relative to β-actin using the comparative C_T method (User Bulletin no. 2, PE Biosystems, UK). Data was ln-transformed prior to statistical analysis to equalise the variance between groups and data compared by 2-way ANOVA for effects of gender and gonadal status. Post-hoc comparisons were conducted using Tukey's test with a significance threshold of $P < 0.05$.

To examine relationships between the levels of mRNA expression of the muscarinic receptors studied and the ability of the detrusor to contract when stimulated with carbachol (chapter 4), mRNA expression levels of each muscarinic receptor for each animal studied were plotted against maximal contraction of isolated strips of detrusor muscle from that

individual where available (as grams of tension produced per mg of wet tissue) and tested for a correlation using Spearman's test with a significance threshold for P (two tailed) < 0.05.

8.3 Results

The mean age and weight of the 75 canines included in this study, 27 entire males (ME), 16 neutered males (MN), 18 entire females (FE), 11 neutered females (FN), and 3 neutered females known to suffering from acquired urinary incontinence (FN AUI) are presented in Table 8-1. There was no statistical difference between the mean ages and weights of the four groups.

	ME (n=27)	MN (n=16)	FE (n=18)	FN (n=11)	FN AUI (n=3)
Age (years)	5.6 ± 0.7	5.7 ± 1.0	6.1 ± 1.0	6.1 ± 0.9	4.3 ± 2.0
Weight (kg)	23.7 ± 1.8	26.9 ± 3.0	21.9 ±2.2	22.4 ± 2.8	17.9 ± 5.0

Table 8-1. Mean ± s.e.mean values for age and weight in groups of entire and neutered male and female canines (ME, MN, FE and FN respectively) plus neutered females known to be suffering from acquired urinary incontinence (FN AUI). n = number of animals.

8.3.1 PCR for M_1, M_2 and M_3

Actin was used as the housekeeping gene, to provide a positive control for mRNA extraction and the primers used are shown in table 8-2. Ventricular and atrial tissue, as well as bladder tissue, was used in initial studies as the M_3 receptor protein has previously been identified within this tissue in the canine (Shi *et al.*, 2004; Shi *et al.*, 1999). The primers that were found to be specific for the M_1 and M_3 receptors are shown in tables 8-3 and 8-4 respectively.

β-Actin	
Forward Primer	5’ – TCC TTC CTG GGC ATG GAA TC – 3’
Reverse Primer	5’ – GGG CGC GAT GAT CTT GAT CT – 3’

Table 8-2. β-Actin PCR primers

Canine M_1 Receptor	
Forward Primer	5’ – TGA CCT CAT CAT CGG TAC CTT C – 3’
Reverse Primer	5’ – ATT CTC CGT CTC CCG GTA GAT – 3’

Table 8-3. M_1 PCR primers

Canine M_3 Receptor	
Forward Primer	5’ – CCT GGC ATA GGT CAT CTC TT – 3’
Reverse Primer	5’ – CTC AGA GCC GAT GTC TTC CTC – 3’

Table 8-4. M_3 PCR primers.

8.3.2 Gels

PCR products were run against standard ladders to allow estimation of the number of base pairs in any resultant bands. Bands of the correct estimated sizes were seen for actin (200bp), M_1 (600bp) and M_3 (500bp) using the primers above.

Bands of the expected size for the M_3 receptor and actin were visualised in atrial, ventricular and bladder samples (Fig. 8-1), and in bladder samples only for the M_1 receptor (data not shown).

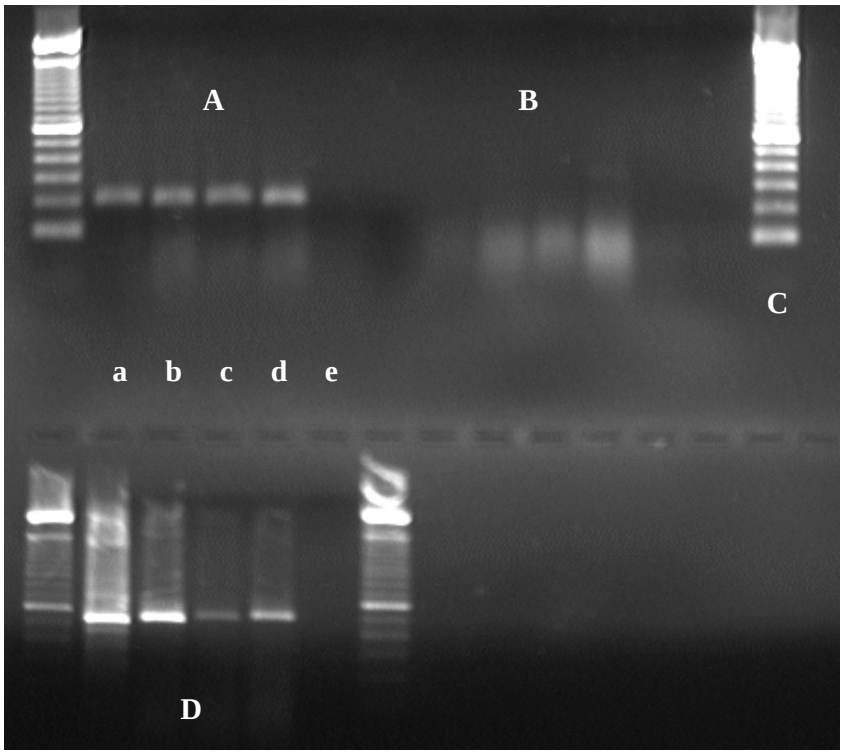

Figure 8-1. Picture of a gel showing the actin bands (A), standard ladder (C) and M_3 bands (D), where a is an atrial sample, b is ventricular, c and d are bladder samples and e is a negative control. B was the result obtained with an additional set of potential M_3 primers that were shown to be non-specific for the target gene.

8.3.3 Sequences

To confirm that the bands observed were the receptors of interest, the bands were excised, purified and sequenced as described in protocols 8.2.7 and 8.2.8 respectively. The consensus sequences for M_1 and M_3 obtained from bladder tissue (and ventricular tissue in the case of M_3) using the forward and reverse primers previously described are shown in tables 8-5 and 8-6 respectively. When run against the entire BLAST database they were found to match the predicted canine M_1 and M_3 sequences and were similar, but not identical to, the identified M_1 and M_3 receptor sequences in other species (≥90% homology in the pig, ≥80% homology in the cow, rat and mouse).

TGC	TCA	TGG	GCC	ACT	GGG	CGC	TGG	GCA	CAC	TGG	CCT
GCG	ACC	TCT	GGC	TGG	CCC	TGG	ACT	ACG	TGG	CTA	GCA
ATG	CCT	CCG	TCA	TGA	ACC	TGC	TGC	TCA	TCA	GTT	TTG
ACC	GCT	ACT	TCT	CCG	TGA	CCC	GGC	CCC	TGA	GCT	ACA
GAG	CCA	AGC	GCA	CAC	CCC	GCC	GGG	CAG	CCC	TGA	TGA
TCG	GCC	TGG	CCT	GGC	TGG	TCT	CCT	TCA	TCC	TCT	GGG
CCC	CGG	CCA	TCC	TCT	TTT	GGC	AGT	ACC	TGG	TAG	GGG
AGC	GGA	CAG	TGC	TGG	CCG	GGC	AGT	GCT	ACA	TCC	AGT
TCC	TCT	CCC	AAC	CCA	TCA	TC					

Table 8-5. Partial sequence of M_1 receptor in the canine.

CTC	ATT	CAG	TTC	CTC	AGC	GAG	CCC	ACC	ATC	ACG	TTC
GGC	ACG	GCC	ATC	GCT	GCC	TTC	TAT	ATG	CCC	GTC	ACC
ATC	ATG	ACT	ATT	TTA	TAC	TGG	AGG	ATC	TAT	AAG	GAG
ACC	GAG	AAA	CGT	ACC	AAA	GAG	CTT	GCC	GGG	CTG	CAG
GCG	TCT	GGG	ACG	GAA	GCA	GAG	GCC	GAG	AAC	TTC	GTC
CAC	CCC	ACA	GGC	AGC	TCT	CGA	AGC	TGC	AGC	AGC	TAT
GAG	CTC	CAG	CAG	CAG	AGC	CTG	AAA	CGC	TCG	GCC	AGG
AAG	AAG	TAC	GGC	CGC	TGC	CAC	TTC	TGG	TTT	GCC	ACC
AAG	AGC	TGG	AAG	CCC	AGC	ACC					

Table 8-6. Partial sequence of M_3 receptor in the canine.

8.3.4 Real Time PCR

Primers and probes for Real Time PCR were designed with reference to the sequences above, and those of the β-actin and the previously published canine M_2 receptor (Shi *et al,*. 2004) in the BLAST database. The primers and probes designed and used in Real Time for β-actin, the M_1, M_2 and M_3 receptors are shown in tables 8-7, 8-8, 8-9 and 8-10 respectively.

	β-actin
Forward Primer	5' – GCC CTG AGG CTC TCT TCC A – 3'
Reverse Primer	5' – GGA ATT GAA GGT AGT TTC GTG ATT – 3'
Probe	5' – CCT TCC TTC CTG GGC ATG GAA TCC – 3'

Table 8-7. β–actin rtPCR primers and probe.

	Canine M_1 receptor
Forward Primer	5' – TCA TCA GTT TTG ACC GCT ACT TCT – 3'
Reverse Primer	5' – GGG TGT GCG CTT GGC TC – 3'
Probe	5' – CGT GAC CCG GCC CCT GAG CTA C– 3'

Table 8-8. Canine M_1 receptor rtPCR primers and probe.

	Canine M_2 receptor
Forward Primer	5' – GAT GGC CTG GAG CAC AAC A – 3'
Reverse Primer	5' – ACA CAG TTT TCG GTC ACA GCA T – 3'
Probe	5' – TCC AGA ATG GCA AAG CCC CCA GA – 3'

Table 8-9. Canine M_2 receptor rtPCR primers and probe.

	Canine M_3 Receptor
Forward Primer	5' – CTT TCT ATA TGC CCG TTC ACC – 3'
Reverse Primer	5' – TGG TAC GTT TCT CGG TCT CCT – 3'
Probe	5' – TGA CTA TTT TAT ACT GGA GGA TCT A – 3'

Table 8-10. Canine M_3 receptor rtPCR primers and probe.

To validate the use of the reagents and allow use of the comparative Ct methods for analysis of gene expression, with these primer probe combinations, the efficiency of the amplification of the primer probe combinations was assessed with serial dilutions of the input amount of cDNA. The results are shown in Fig. 8-2a, b and c for M_1, M_2 and M_3 respectively.

To allow meaningful comparisons of mRNA expression and correct for differences in the amount of tissue extracted it is necessary to use relative gene expression whereby the expression of each gene is compared to a housekeeping gene, in this case β-actin, which is known to be evenly expressed within the tissue to be studied. To assess the suitability of any pair of housekeeping gene and gene of interest, it is first necessary to ensure that the rate of amplification within the qPCR conditions used is similar. This is achieved by comparing gene amplification from different dilutions of a stock mRNA sample. For this, standard dilutions of a sample using the above primers and probes for β-actin and the gene of interest were made and run using Real Time PCR protocol 8.2.9. If mRNA amplification efficiencies are equal a plot of ΔCt versus dose should produce a horizontal line. The results obtained using the real time PCR primers and probes for β-actin and canine M_1, M_2 and M_3 receptors used for subsequent analysis are shown in Fig. 8-3.

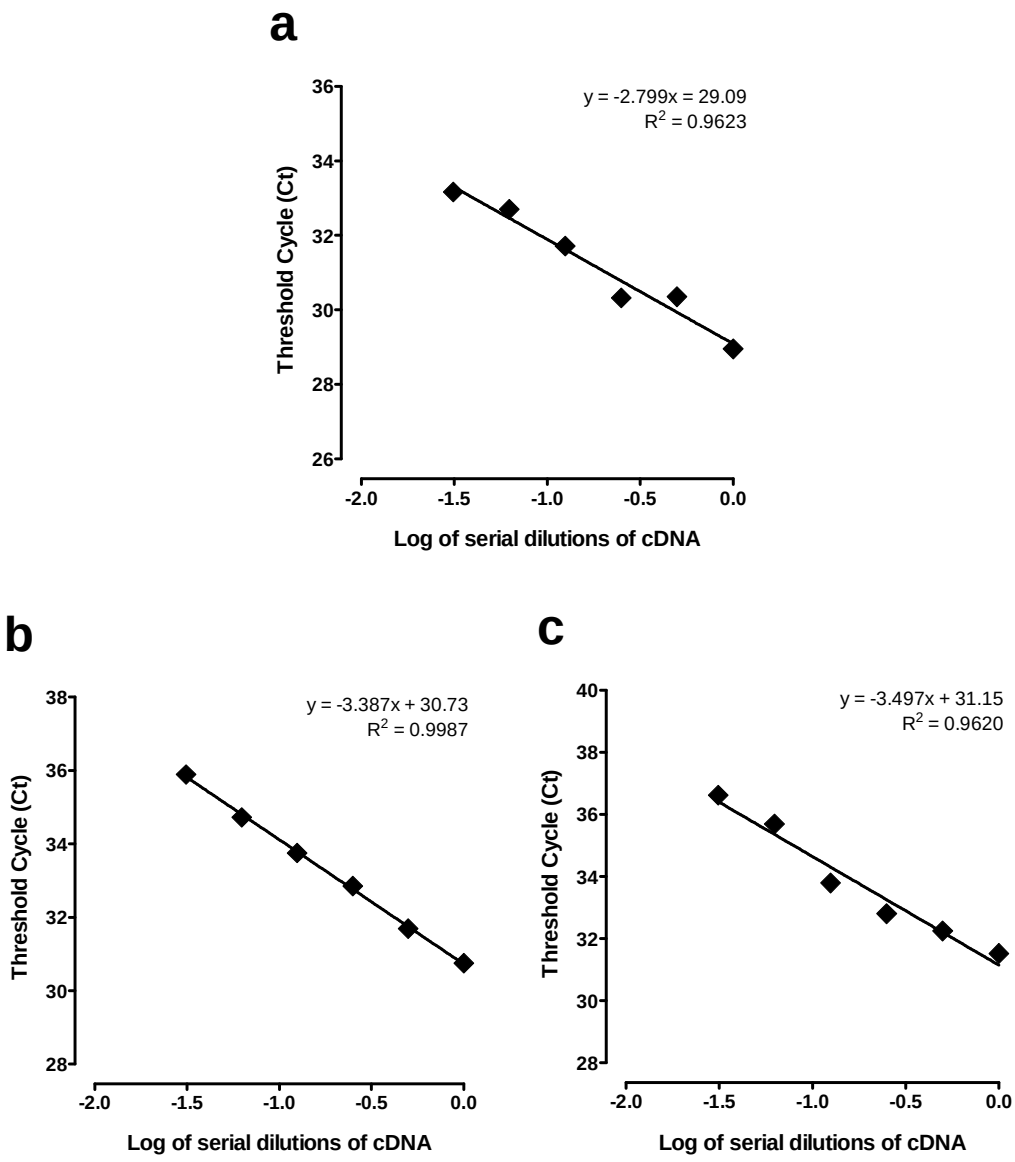

Figure 8-2. Linearity assessment of mRNA expression for a., M_1, b, M_2 and c, M_3 receptor, using serial dilutions of input cDNA reverse transcribed from canine urinary bladder.

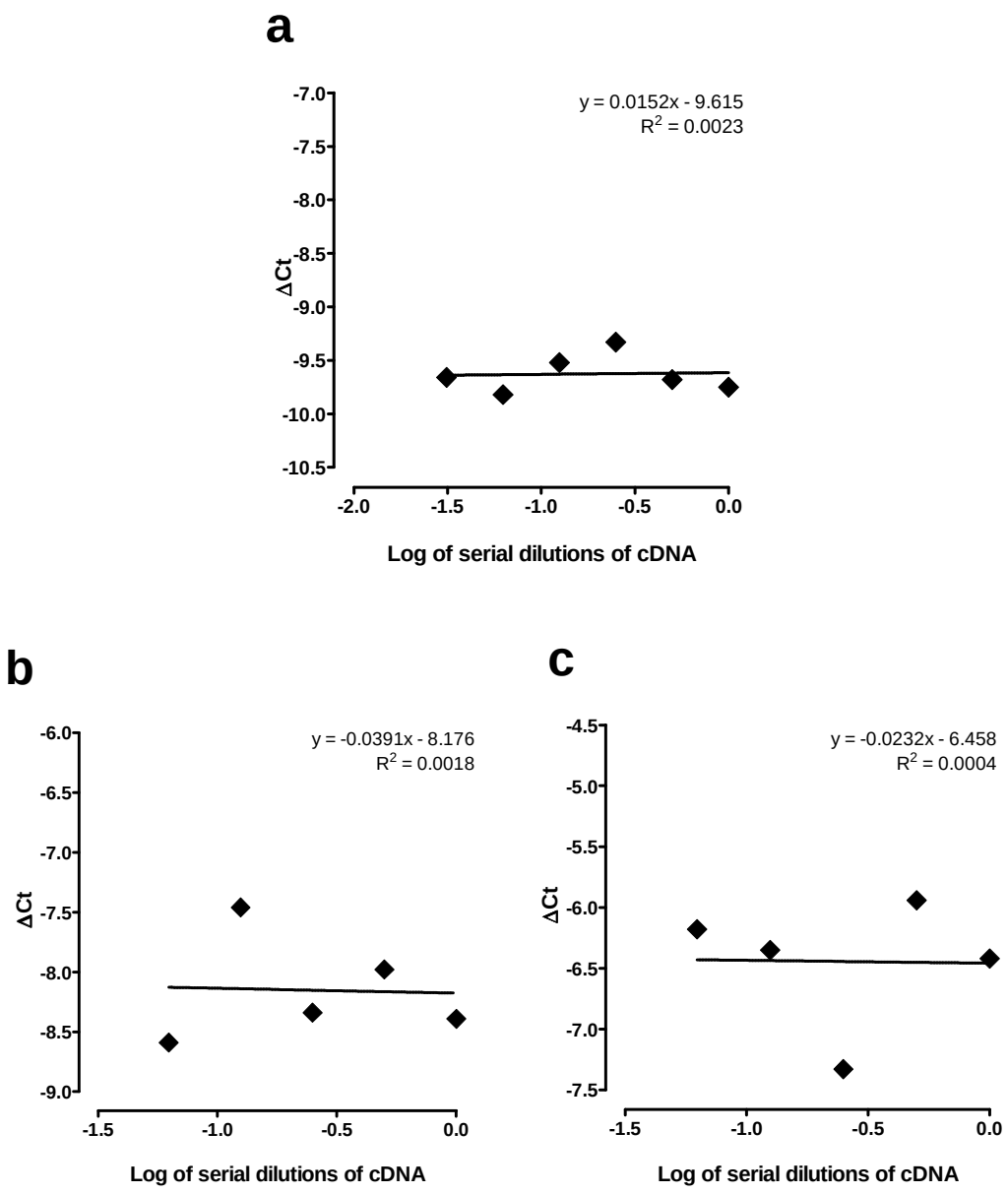

Figure 8-3. Gene amplification assessment of a, M_1, b, M_2 and c, M_3 receptor against the housekeeping gene β-actin.

The mean levels of mRNA expression for M_1, M_2 and M_3 receptor are shown in Figures 8-4a, b and c respectively. 2-way ANOVA indicated significant effects of gonadal status on the expression of the mRNAs for all muscarinic receptors, with neutered animals having higher expression levels than their entire counterparts ($p<0.05$). There was no interaction between the effects of gonadal status and gender on the expression of mRNA for any receptor subtype. A significant effect ($P<0.05$) of gender on the mRNA expression levels for M_1 receptor was also observed, with female animals having higher levels of expression than their male counterparts, regardless of gonadal status. The mRNA expression levels for the M_2 receptor for the females identified as suffering from acquired urinary

incontinence were the highest of all the animals in the study and fell above the 95% CI for the continent neutered female group (3.16- 3.27), however, this difference was not statistically significant (fig. 8-4b). mRNA expression for the M_1 and M_3 receptor subtypes did not differ in the female neuters that suffered from acquired urinary incontinence as the levels of expression seen in these three animals fell within the 90% CI for the continent neutered female groups.

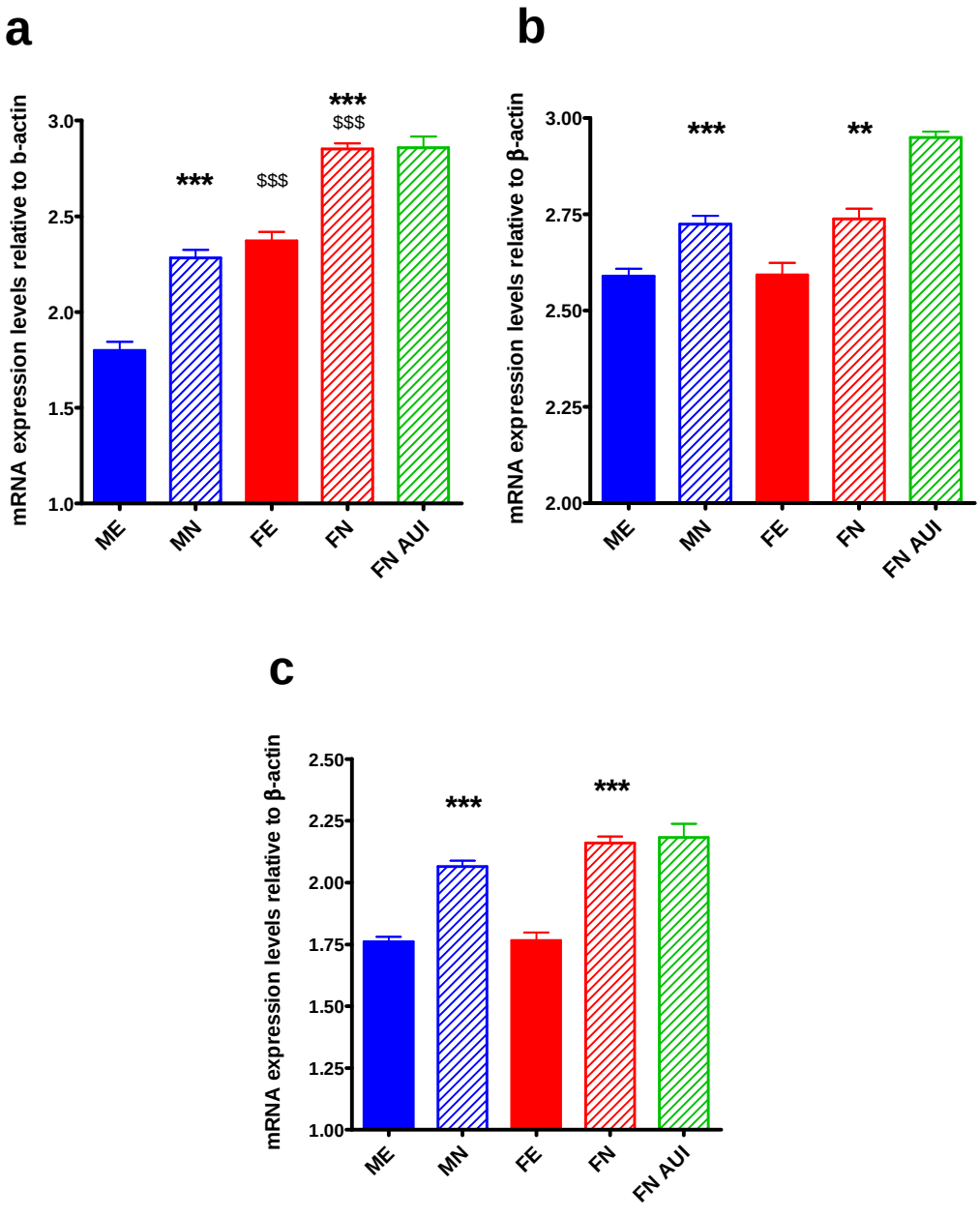

Figure 8-4. Mean (± s.e.mean) mRNA expression for: a, M_1 receptor, b, M_2 receptor and c, M_3 receptor, in entire and neutered male and female canines (ME, MN, FE and FN respectively) and neutered female canines identified as suffering from acquired urinary incontinence (FN AUI). mRNA values are logarithmically transformed. *** P<0.001, ** P<0.01 compared entire of same gender. $$$ P<0.001 compared to male animals of same gonadal status.

Carbachol induced dose dependant contraction of the isolated strips of detrusor muscle from this specific subset of animals, as was the case in the larger study population described in chapter 4. As in the previous study, maximal response was significantly decreased ($P<0.01$) in neutered compared to entire canines of either gender (Figure 8-5). Sensitivity of strips to carbachol, as measured by Log EC_{50} values, was also decreased in neutered compared to entire animals ($P<0.01$). 2 way ANOVA revealed there was no effect of gender and no interaction between gender and gonadal status. The group of animals identified as suffering from acquired urinary incontinence had the lowest maximal contraction of all animals studied, although this was not considered significant using ANOVA analysis.

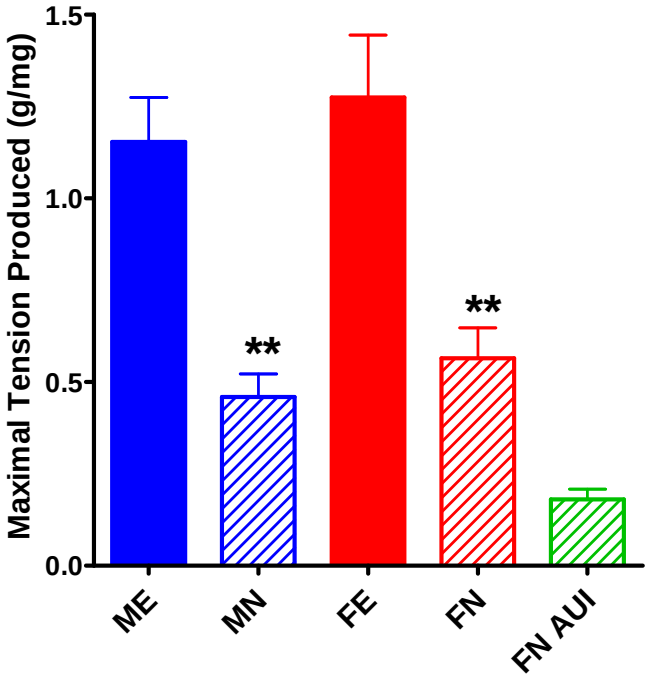

Figure 8-5. Mean (± s.e.mean) maximal tension values (g/mg wet tissue) in response to carbachol in isolated strips of detrusor muscle from entire and neutered male and female canines (ME, MN, FE and FN respectively) and neutered female canines identified as suffering from acquired urinary incontinence (FN AUI). Data is for canines for which both maximal contraction to carbachol and mRNA expression levels for muscarinic receptors are available. ** P<0.01 compared entire of same gender

Statistical analysis revealed a significant negative correlation between the maximal tension produced by detrusor muscle strips and M_1 ($P<0.05$, $r=-0.362$), M_2 ($P<0.01$, $r=-0.579$) and M_3 ($P<0.05$, $r=-0.678$) receptor mRNA expression (Figures 8-6a, b and c respectively).

Multivariant analysis of the levels of expression of mRNA for the muscarinic receptors and the maximum tension produced by detrusor muscle strips indicated no significant effect of age or weight of the animal on the results.

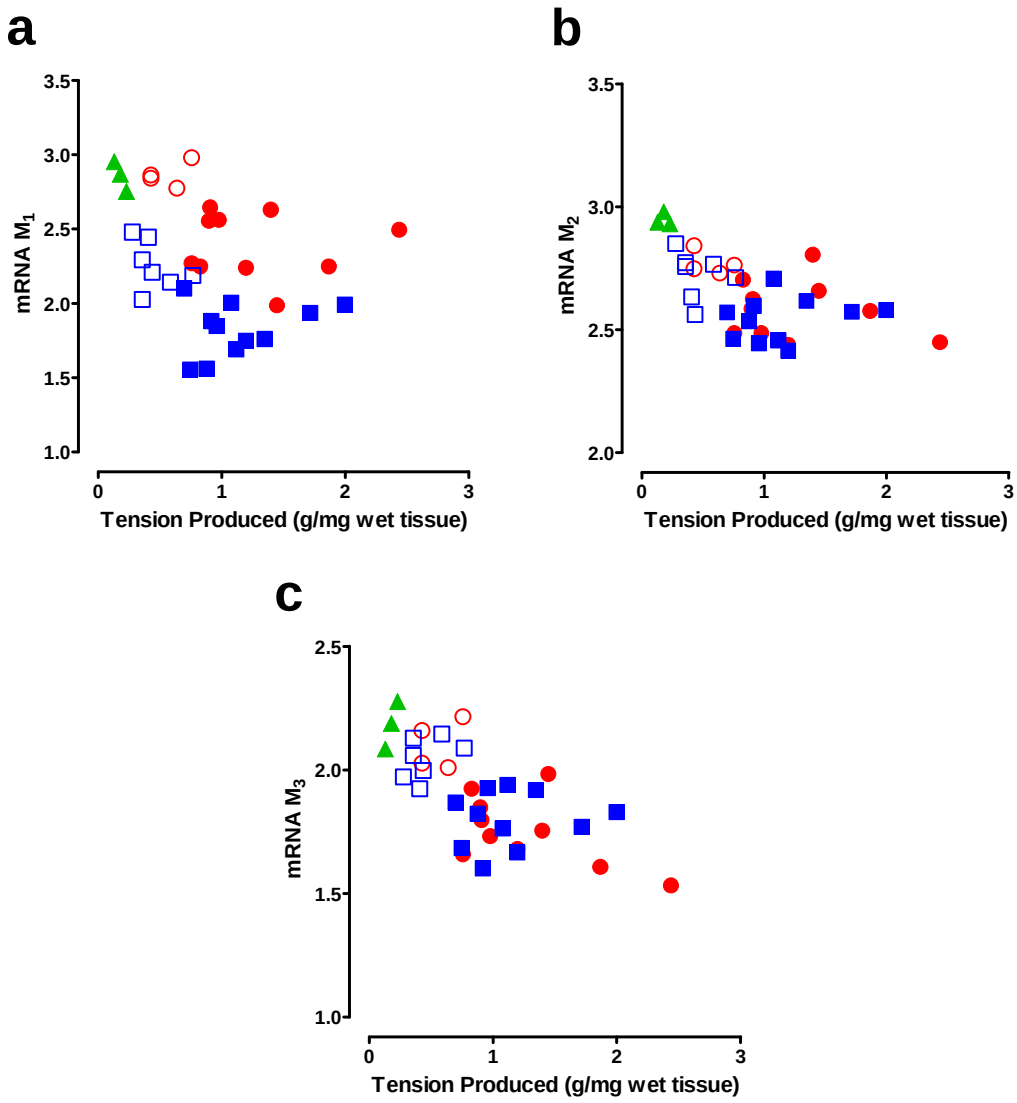

Figure 8-6. Correlation between maximal tensions produced by detrusor muscle strips *in vitro* in response to muscarinic stimulation and mRNA expression levels for: a, M_1 receptor ($P<0.05$, $r=-0.362$), b, M_2 receptor ($P<0.001$, $r=-0.579$) and c, M_3 receptor ($P<0.05$, $r=-0.678$). ■ entire male, □ neutered male, ● entire female, ○ neutered female, ▲ neutered female known to be suffering from acquired urinary incontinence.

8.4 Discussion

This study is the first to report the presence of, and levels of expression for, mRNA for the M_1, M_2 and M_3 receptors in the canine urinary bladder. The results also show that the amplicons isolated for the canine M_1 and M_3 receptors in this study have 100% homology with the predicted canine sequence and are similar to the identified M_1 and M_3 receptor sequences in other species ($\geq$ 90% homology in the pig, $\geq$ 80% homology in the cow, rat and mouse). The results demonstrate that mRNA expression of M_1, M_2 and M_3 receptors within the canine urinary bladder can be quantified by real time PCR, relative to β-actin. The present work demonstrates that mRNA for these receptors is expressed in canine urinary bladder and that a significant relationship exists between levels of mRNA expression and tissue contractility. Furthermore, this study indicates a significant effect of neutering on mRNA expression levels for the M_1, M_2 and M_3 receptors.

This study has shown that neutered animals have significantly higher expression levels of mRNA for all three receptors than their entire counterparts, regardless of gender, and that female animals have higher expression levels of M_1 receptor mRNA than their male counterparts. For those animals known to be suffering from acquired urinary incontinence the levels of expression for the M_2 receptor mRNA were the highest of all animals studied, however the expression for the M_1 and M_3 receptor mRNAs were similar to that of continent neutered animals. This study has also demonstrated an inverse relationship between receptor mRNA expression and contractility of the bladder to muscarinic stimulation *in vitro* for all receptors, with animals known to be suffering from acquired urinary incontinence having the lowest contractility and highest M_2 receptor mRNA expression of all animals studied.

There have been no reported studies investigating the relationship between muscarinic receptor mRNA expression levels and impaired contractility of the detrusor in any species; therefore, accurate comparisons with the results of this study are not possible. However, a number of other studies in various species have looked at the expression levels of the muscarinic receptor subtypes within the bladder, their functional properties and how these expression levels and properties change in certain disease states. This allows the formation of hypotheses as to how the muscarinic receptors may mediate function in the canine urinary bladder and also helps aid understanding of the potential significance of the changes in muscarinic receptor mRNA expression levels demonstrated in this study.

The M_1 receptor has been variably reported in the detrusor muscle of rats (Braverman *et al.*, 1998a) and humans (Kondo *et al.*, 1995), however, a further study has not found this receptor within the detrusor of human, rabbit, rat or guinea-pig bladder (Wang *et al.*, 1995) and it is not considered to play a primary role in detrusor contraction (Chess-Williams, 2002). In contrast, the M_1 receptor has been described as occurring on the pre-synaptic nerve terminals in a number of species where they are thought to facilitate transmitter release and enhance contraction of the bladder (Braverman *et al.*, 1998a; Somogyi *et al.*, 1999; Somogyi *et al.*, 1994). Due to the limitations of this study it is not possible to determine if the M_1 mRNA detected in the bladder of canines is presynaptic or on the detrusor muscle itself, however, evidence from studies in other species suggest that it is most likely to be presynaptic in origin. If this is indeed the case, it is possible that the M_1 receptor is up-regulated in neutered animals in an attempt to facilitate ACh release and thereby enhance contractile force of the detrusor which has been decreased due to another, as yet unidentified mechanism.

The results show an increase in the expression of the mRNA for the M_2 receptor in neutered animals, with the incontinent animals having the highest level of M_2 expression of all animals studied. There are no reported studies looking at levels of M_2 receptor in the bladder of humans suffering from urinary incontinence due to impaired contractility of the detrusor, however M_2 expression has been shown to be increased in other disease states including denervation of the bladder where they are known to contribute significantly to contraction (Braverman *et al.*, 1998b). The M_2 receptor has been shown to dominate within the urinary bladder in all species so far studied (Goepel *et al.*, 1998; Wang *et al.*, 1995; Yamanishi *et al.*, 2000) and it is described mainly on the detrusor muscle where it is thought to play a role in bladder contraction, as demonstrated by M_2 knockout mice that have decreased bladder responsiveness (Stengel *et al.*, 2000). A study in pigs has shown that the contractile response of the M_2 receptor to cholinergic stimulation may be smaller than that of the M_3 receptor and that the sensitivity of the M_2 receptor may also be less than that of the M_3 receptor. This could be significant in this study if the M_2 receptor population is increasing to play a greater role in contraction of the bladder in neutered animals, as suggested by the data demonstrated in chapter 5 of this thesis in which it was shown that the M_2 receptor is the primary receptor responsible for detrusor muscle contraction in neutered canines *in vitro*, then the contraction produced may be reduced in comparison to that produced by a healthy individual with a contraction mediated by the M_3 receptor. This possibility is supported by the data from animals known to be suffering from acquired urinary incontinence which had the highest M_2 receptor expression yet the lowest contractility and sensitivity of the bladder to muscarinic receptor stimulation.

The M_3 receptor has been reported on the detrusor muscle of all species so far studied, and is considered the primary receptor responsible for mediating detrusor contraction in healthy individuals (Braverman *et al.*, 1998a; Braverman *et al.*, 1998b; Chess-Williams, 2002; Chess-Williams *et al.*, 2001; Choppin *et al.*, 2001; Goepel *et al.*, 1998; Ikeda *et al.*, 1999; Longhurst *et al.*, 2000; Sellers *et al.*, 2000; Wang *et al.*, 1995). Despite its role in mediating contraction it has previously been reported that the M_3 receptor does not predominate within the detrusor (Wang *et al.*, 1995) and the results of this canine study are in agreement with this. Due to its role in micturition it might reasonably be assumed that decreased contractility of the detrusor muscle would be accompanied by decreased expression of M_3 receptor, however, the results of this present study demonstrate that decreased contractility of the detrusor muscle is associated with an increased M_3 mRNA expression in neutered canines. This apparent paradox may be due to the M_3 receptor becoming dysfunctional (Eglen *et al.*, 1994), however, it is also conceivable that the increased M_3 receptor mRNA expression demonstrated in this study is not reflected by increased M_3 protein expression as described by Braverman *et al.* (Braverman *et al.*, 2006) who reported a lack of correlation between mRNA and protein expression for this particular receptor. It can therefore be hypothesised that the increase in M_3 mRNA expression seen in neutered canines in this study is a compensatory response to the decreased detrusor contractility brought about by an as yet unidentified mechanism that may involve transcription or translation of the M_3 receptor itself.

In conclusion this study has shown that neutering a canine, regardless of gender, is associated with an increase in the levels of mRNA for M_1, M_2 and M_3 receptors in the canine urinary bladder. Furthermore, these results show that there is a negative correlation between increased mRNA expression of these receptors and decreased contractility of the detrusor to muscarinic stimulation *in vitro*. It is hypothesised that the changes in mRNA expression for these receptors may be linked to the development of post neutering acquired urinary incontinence and this hypothesis is supported by data from a limited number of neutered female canines identified as suffering from acquired urinary incontinence which had the highest levels of M_2 receptor mRNA expression and the lowest contractility to muscarinic stimulation of all the animals studied. Further studies are required to investigate the protein expression levels of these receptors, as well as their function in mediating bladder contractility before the exact role of these receptors, and their suitability as therapeutic targets in acquired urinary incontinence in the bitch can be determined.

9 Neutering affects mRNA expression levels for the LH- and GnRH- receptors in the canine urinary bladder.

9.1 Introduction

Urinary incontinence is a debilitating and so far incurable condition that causes significant welfare problems for affected canines. Urinary incontinence is seen most frequently in neutered female canines where the condition is termed acquired urinary incontinence, and is thought to be due to insufficient urethral closure pressure and / or abnormal bladder storage function (Arnold, 1997; Nickel *et al.*, 1997).

The previously reported *in vitro* studies have shown that neutering a canine of either gender leads to a decrease in maximal contractile response of the detrusor muscle to both muscarinic and electrical field stimulation, as well as a decreased sensitivity to muscarinic stimulation (Coit *et al.*, 2008). These decreases in contractile function *in vitro* are similar to those reported in post menopausal women who suffer from urinary incontinence due to impaired contractility of the bladder (Andersson *et al.*, 2004a; Elbadawi *et al.*, 1993a; Zhu *et al.*, 2001). It is hypothesised that this decrease in bladder function may be due to, or exacerbated by, hormone mediated effects. In addition a common element between the two identified susceptible populations is that, in both spayed bitches and post menopausal women, there is a deficiency of endogenous gonadal steroid hormones. This deficit in turn decreases or removes the normal negative feedback to the hypothalamo-pituitary axis, and results in greatly increased production and secretion of GnRH from the hypothalamus leading to increased secretion of the pituitary gonadotrophins LH and FSH (Burger, 1996; Olson *et al.*, 1992; Reichler *et al.*, 2004; Reichler *et al.*, 2005b; Wise, 1999). The post neutering increase in plasma concentrations of LH and FSH has been demonstrated to have a direct relationship with the development of urinary incontinence in the bitch *(Reichler et al.*, 2005a) and a recent paper has described the clinical use of GnRH analogues to decrease LH and FSH concentrations in neutered female canines suffering from acquired urinary incontinence with a positive clinical outcome i.e. clinical continence, or an improvement in continence, during the treatment period (Reichler *et al.*, 2006a; Reichler *et al.*, 2003). This result would suggest that the increase in the concentrations of either GnRH and/or the pituitary gonadotrophins could be a causative factor for acquired urinary incontinence in the bitch.

While GnRH and the gonadotrophins are classically thought of as reproductive hormones of the hypothalamo-pituitary-gonadal axis, the presence of their receptors in tissues such as skin (Welle *et al.*, 2006), reproductive tract, prostate and mammary gland (Fields *et al.*, 2004; Ziecik *et al.*, 2005) as well as the urinary bladder (Ponglowhapan *et al.*, 2007b; Reichler *et al.*, 2007; Tao *et al.*, 1998) would suggest more widespread actions. In particular the presence of receptors within the urinary tract could lead to local effects of elevated GnRH and gonadotrophin concentrations. As tissue action and function rely upon the presence of specific receptors, it is possible that changes in either absolute receptor numbers or receptor-ligand concentration could influence the function of, or stimulate structural changes within a tissue. In this regard a study in women has reported a decrease in LH- receptor numbers in the bladders of post- compared to pre-menopausal women (Tao *et al.*, 1998) and a number of recent studies in the canine have reported the mRNA levels of receptors within the canine bladder (Ponglowhapan *et al.*, 2007a; Reichler *et al.*, 2007) but the results have been contradictory as both a decrease and no change were seen following neutering in these two studies. To the authors knowledge there have been no reported studies looking at mRNA levels for the LH, FSH or GnRH receptors in the bladders of bitches known to be suffering from acquired urinary incontinence. There have also been no studies reported looking at the correlation between detrusor contractility, a factor known to be involved in urinary incontinence *(Elbadawi et al., 1993a; Elbadawi et al.*, 1993b) and receptor mRNA expression levels within the urinary bladder.

This study tested the hypothesis that the expression levels of GnRH-, LH- and FSH-receptor mRNA in the canine urinary bladder will be altered by neutering and that these changes will be most extreme in neutered bitches suffering from acquired urinary incontinence. Furthermore the hypothesis that there will be a correlation between the expression of the mRNAs for these receptors and maximal contractility of the bladder was tested.

9.2 Materials and Methods

9.2.1 Animals

The study was approved by The University of Glasgow Veterinary School's ethical review committee. Tissue from a total of 78 canines was included in the study. The study population had a mean age of 6.0 ± 0.5years (range 1-16 years) and a mean weight of 22.7 ± 1.3kg (range 8-50kg). The canines were split into five groups depending on gender, gonadal status and incidence of acquired urinary incontinence: entire and neutered males, entire and neutered females, plus neutered females known to be suffering from acquired urinary incontinence. The majority of canines were cross bred, with no pedigree breeds appearing more than once. In all cases, tissue was collected within 2 hours of euthanasia (intravenous overdose of pentobarbatone) with full informed owner consent, for reasons other than scientific investigation. The majority of animals had been euthanized for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. None of the animals used in the study were receiving medical treatment in the 7 days prior to euthanasia and there was no evidence of previous medical therapy in at least the month prior to this. A full gross post mortem of the reproductive and urinary tract was performed before tissue harvesting and any animals with a history or signs of gross pathological urinary, neurological or reproductive tract disease (e.g. tumours, cystitis, pyometra) were excluded from the study.

To ensure best use of tissue available and to allow comparisons between different parameters a number of the animals included in this study were also included in the studies described elsewhere in this thesis.

9.2.2 Preparation of Tissue

Full thickness biopsies (2cm by 2cm) were collected from the area of the bladder dome. Ovarian tissue as well as skin biopsies, from the shoulder and groin regions were collected from a subset of animals included in the study, as a control for FSH receptor mRNA expression (Welle *et al.*, 2006). Samples were immediately frozen and stored at -80°C until required for mRNA studies.

9.2.3 mRNA Extraction

The same protocol as described in 8.2.3 was used in this study.

9.2.4 Reverse Transcription

The same protocol as described in 8.2.4 was used in this study.

9.2.5 Real Time PCR

The primers and probes for the three receptor mRNAs were as described by Welle *et al* (Welle *et al.*, 2006), sourced from Eurogentech (Southampton, UK).

The protocol used was as described in 8.2.9.

9.2.6 Statistical Analysis

Results for mRNA expression levels were analysed against both age and weight using multivariant analysis with $P \leq 0.05$ considered significant.

Results are given as mRNA expression levels relative to β-actin using the comparative C_T method (User Bulletin no. 2, PE Biosystems, UK). Data was ln-transformed prior to statistical analysis to equalise the variance between groups and data compared by 2-way ANOVA for effects of gender and gonadal status. Post-hoc comparisons were conducted using Tukey's test with a significance threshold of $P < 0.05$.

To examine relationships between the levels of mRNA expression of the hormone receptors studied and the ability of the detrusor to contract when stimulated with carbachol (chapter 4), mRNA expression levels of each hormone receptor were plotted against maximal contraction (as grams of tension produced per mg of wet tissue) and tested for a correlation using Spearman's test with a significance threshold for P (two tailed) < 0.05.

9.3 Results

The mean age and weight of the 78 canines included in this study, 27 entire males (ME), 17 neutered males (MN), 18 entire females (FE), 13 neutered females (FN), and 3 neutered females known to suffering from acquired urinary incontinence (FN AUI) are presented in Table 9-1. There was no statistical difference between the mean ages and weights of the four groups.

	ME (n=27)	MN (n=17)	FE (n=18)	FN (n=13)	FN AUI (n=3)
Age (years)	5.6 ± 0.7	5.8 ± 0.9	6.1 ± 1.0	6.5 ± 1.0	4.3 ± 2.0
Weight (kg)	23.7 ± 1.8	27.4 ± 3.2	21.9 ±2.2	21.1 ±3.1	17.9 ± 5.0

Table 9-1. Mean ± s.e.mean values for age and weight in groups of entire and neutered male and female canines (ME, MN, FE and FN respectively) and neutered female animals known to be suffering from acquired urinary incontinence. n = number of animals (FN AUI).

9.3.1 Validation of qPCR primer probes combinations to study hormone receptor gene expression

To validate the use of the reagents and allow use of the comparative Ct methods for analysis of gene expression, with these primer probe combinations, the efficiency of the amplification of the primer probe combinations was assessed with serial dilutions of the input amount of cDNA. The results are shown in Fig. 9-1a and b for LH and GnRH respectively.

As in chapter 8, amplification efficiencies were calculated for each of the receptors of interest against the housekeeping gene β-actin, as shown in Fig 9-2.

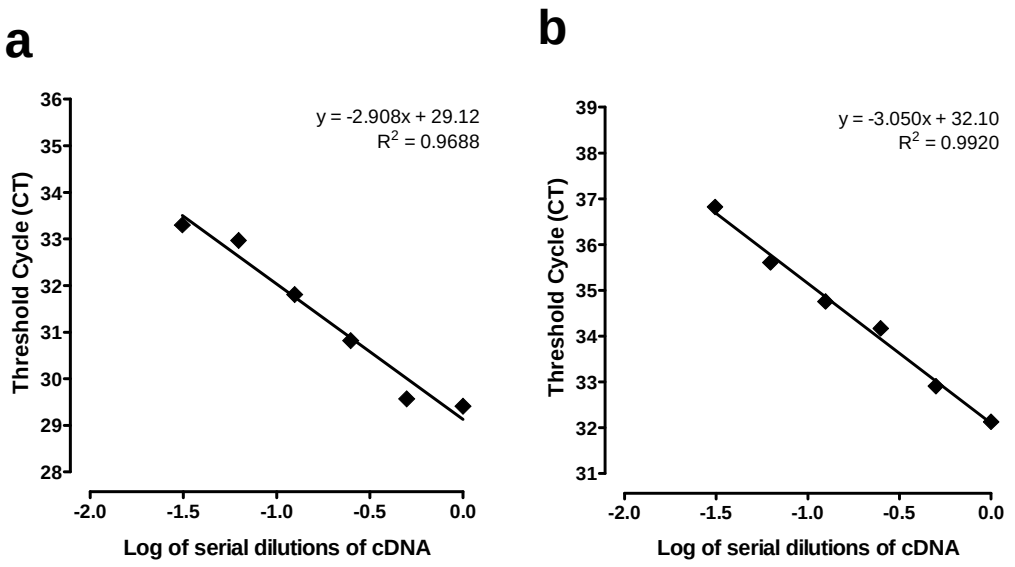

Figure 9-1. Linearity assessment of mRNA expression for a, LH receptor and b, GnRH receptor, using serial dilutions of input cDNA reverse transcribed from canine bladder.

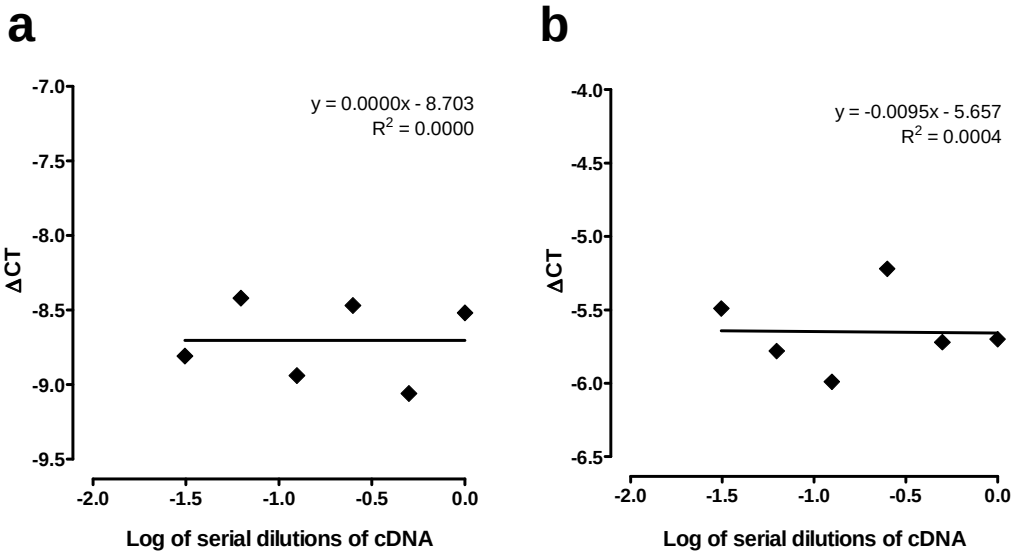

Figure 9-2. Gene amplification assessment of a, LH receptor and b, GnRH receptor against the housekeeping gene β-actin.

9.3.2 Comparison of LH and GnRH receptor gene expression between groups.

The mean levels of mRNA expression for LH and GnRH receptor are shown in Figures 9-3a and b respectively. 2-way ANOVA indicated significant effects of gonadal status on the expression of the mRNAs for both LH- and GnRH-receptor, whilst there was an additional effect of gender on the expression of LH-receptor mRNA. There was no statistically significant interaction between the effects of gonadal status and gender. Neutering was associated with significantly ($p<0.01$) higher levels of mRNA expression for both LH- and GnRH- receptor in both genders. Furthermore, female animals showed significantly ($p<0.05$) greater levels of mRNA expression for LH- receptor, than their male counterparts, regardless of gonadal status (Figure 9-3a).

mRNA expression levels for both LH- and GnRH- receptor for the females identified as suffering from acquired urinary incontinence were the highest of all the animals in the study and fell above the 95% CI for the continent neutered female group, however, the difference between the incontinent and continent neutered females was not statistically significant.

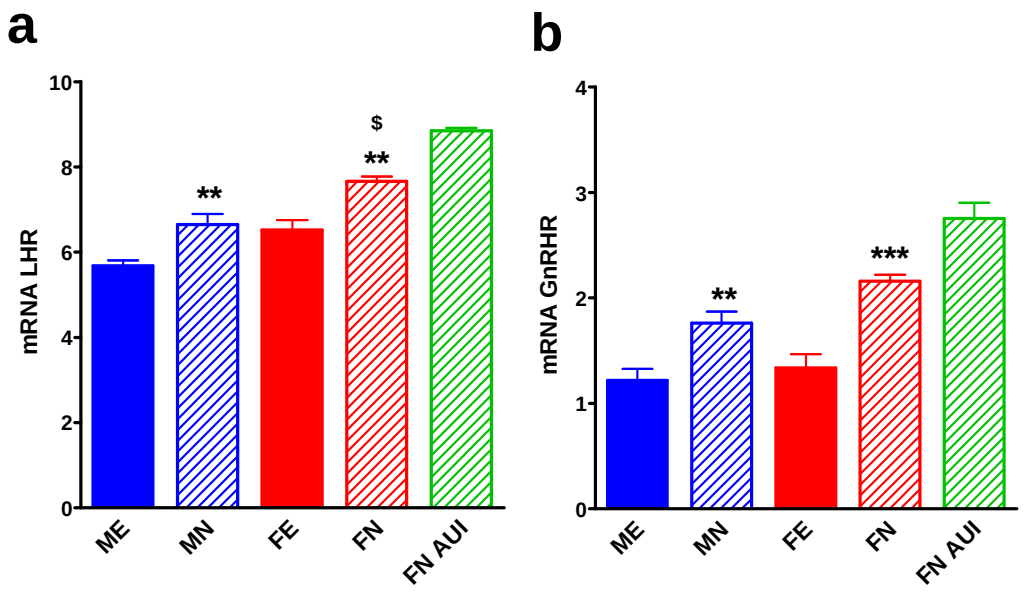

Figure 9-3. Mean (± s.e.mean) mRNA expression for: a**, LH receptor and** b**, GnRH receptor, in isolated strips of detrusor muscle from entire and neutered male and female canines (ME, MN, FE, and FN respectively) and neutered female canines identified as suffering from acquired urinary incontinence (FN AUI). mRNA values are logarithmically transformed. *** P<0.001, ** P<0.01 compared entire of same gender. $ P<0.05 compared to male animals of same gonadal status.**

9.3.3 Real time PCR for FSH receptor mRNA

Real time PCR was performed for the quantification of FSH receptor mRNA expression within samples of urinary bladder. In all cases, expression was found to be below the limits of detection of the assay system. To ensure validity of the primers and probes used, they were tested against samples of canine ovary and skin as described by Welle *et al* (Welle *et al.*, 2006). mRNA for the FSH- receptor was found at very low levels ($3.22E^{-15}$, n=2) in samples of canine skin but was not detectable in samples of canine ovary.

9.3.4 Relationship between receptor mRNA expression and tissue contractility

Analysis of the contractility of the tissue strips for which mRNA levels were also quantified, indicated that as shown previously (Chapter 4), the maximal response in

neutered compared to entire canines of either gender was significantly decreased (P<0.01) and the group of animals identified as suffering from acquired urinary incontinence had the lowest maximal contraction of all animals studied (Figure 9-4).

Statistical analysis revealed a significant negative correlation between the maximal tension produced by detrusor muscle strips and both LH- (P<0.001, r=-0.671) and GnRH- (P<0.001, r=-0.612) receptor mRNA expression (Figures 9-5a and b respectively).

Multivariant analysis of the levels of expression of mRNA for both GnRH- or LH-receptor, and the maximum tension produced by detrusor muscle strips indicated no significant effect of age or weight of the animal on the results.

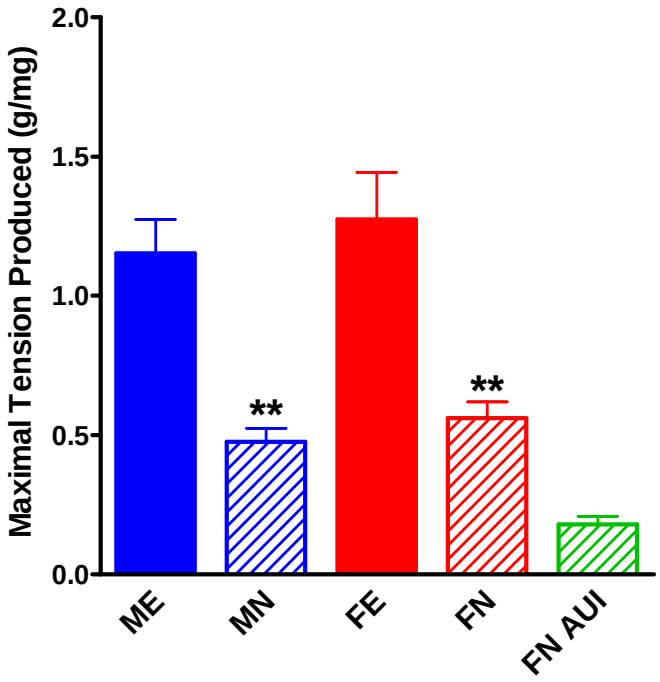

Figure 9-4. Mean (± s.e.mean) maximal tension values (g/mg wet tissue) in response to carbachol in isolated strips of detrusor muscle from entire and neutered male and female canines (ME, MN, FE and FN respectively) and neutered female canines identified as suffering from acquired urinary incontinence (FN AUI). Data is for canines for which both maximal contraction to carbachol and mRNA expression levels for GnRH and LH are available. ** P<0.01 compared entire of same gender.

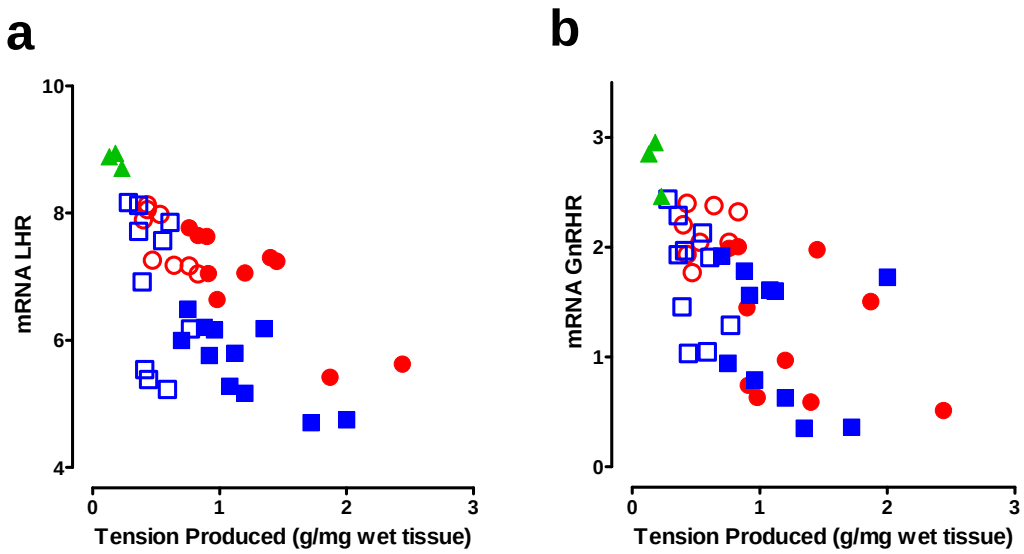

Figure 9-5. Correlation between maximal tensions produced by detrusor muscle strips *in vitro* and mRNA expression levels for: a, **LH receptor (P<0.001, r=-0.671) and** b, **GnRH receptor (P<0.001, r=-0.612).** ■ **entire male,** □ **neutered male,** ● **entire female,** ○ **neutered female,** ▲ **neutered female known to be suffering from acquired urinary incontinence.**

9.4 Discussion

Bladder contractility is thought to play a significant role in the development of clinical signs of urinary incontinence (Andersson *et al.*, 2004a). The results presented in chapter 4 (Coit *et al.*, 2008) showed that the maximal contractile response and sensitivity of isolated strips of detrusor muscle to muscarinic and neurogenic stimulation is significantly decreased in neutered compared to entire canines of either gender and the same result was seen in the subset of animals for which tissue was processed for mRNA comparison in this study. These results are similar to those of women who suffer from urinary incontinence due to impaired contractility of the bladder (Elbadawi *et al.*, 1993a), a condition known to cause decreased speed and magnitude of detrusor contractions during the emptying phase of micturition, often leading to residual urine retention, shortened storage phase and predisposing the patient to cystitis (Resnick *et al.*, 1987). Although impaired bladder contractility has been implicated in urinary incontinence, the exact mechanisms responsible for its development have not been reported. This study tested the hypothesis that changes in reproductive hormones and their receptors post neutering may alter the contractility of the bladder.

The possibility that gonadotrophins could influence bladder function was first suggested in a study which showed that postmenopausal women, who are most likely to suffer from urinary incontinence, had lower numbers of LH mRNA transcripts and receptors in their bladders than their premenopausal counterparts (Tao *et al.*, 1998). As postmenopausal women have a similar hormonal profile to neutered canines and as both post-menopausal and post-neutering urinary incontinence are thought to be hormonally related, it follows that a similar aetiology and pathophysiology may link both conditions. These results clearly show that neutering a canine, regardless of gender, significantly increases levels of mRNA expression for both LH- and GnRH- receptors and that, in general, female animals, regardless of gonadal status have higher levels of mRNA expression for these receptors than their male counterparts. Although the results for female canines are in contrast to the study by Tao *et al.* the results of this present study include age matched groups of healthy entire and neutered animals as well as those known to be suffering from acquired urinary incontinence. The study by Tao *et al*. compared women of two distinct age groups, thus it is not possible in that study to dissect out the individual effects of age or menopausal status on LH receptor mRNA expression and as a number of patients in both groups suffered from urinary incontinence, it is not possible to determine if the observed decrease in LH receptor mRNA expression was linked to predisposition to urinary incontinence. In

addition it should be noted that there are multiple types of urinary incontinence recognised in human medicine hence the observation of incontinence in both pre and post menopausal groups. Thus more detailed analysis of the human data would be required wherein patients were separated into subgroups based on the type of urinary incontinence in addition to their menopausal status. The results of the current study, however, interestingly, suggest that LH- receptor mRNA may actually be increased in animals suffering from acquired urinary incontinence relative to their continent counterparts. These findings support the hypothesis that gonadotrophins have a role in the pathophysiological mechanism that leads to post-neutering urinary incontinence in the bitch (Reichler *et al.*, 2003).

The present findings of canine receptor mRNA levels, however, contrast with those reported by Reichler *et al.* (Reichler *et al.*, 2007) and Ponglowhapan *et al.* (Ponglowhapan *et al.*, 2007a) as they reported either no change or a decrease in mRNA expression for LH and GnRH receptors in neutered canines, respectively. These differences in results may reflect differences in the study populations and conditions. In both of the studies by Reichler *et al.* and Ponglowhapan *et al.* a substantially smaller number of animals were used which may affect the results obtained. In addition in the study by *Reichler et al*. the groups were not age matched and included entire bitches in varying stages of the oestrus cycle, which, if bladder hormone receptor expression is influenced by changes in the reproductive hormones as hypothesised, could significantly affect the results obtained, however, oestrous cycle stage was not included in the analysis. Thus the small sample size and increased variation may have prevented statistical observation of an effect of neutering on LH receptor mRNA expression. The study by Ponglowhapan *et al*. was conducted using animals that had been neutered for a relatively short time (24-40 weeks). As it has been shown that plasma concentrations of FSH and LH continue to increase until week 42 post neutering (Reichler *et al.*, 2004) and that some structural changes may have a chronic time course (Fleischmann *et al.*, 2002) it is possible that the decrease in receptor mRNA expression in that study reflect more acute changes. Indeed it could be hypothesised that if receptor mRNA was produced at a relatively constant level that it should be inversely related to protein expression and thus a decrease in expression could be seen initially as receptor number increases in association with steroid withdrawal, but then levels increase as receptor down regulation results in decreased translation.

A further difference between this study and that of Tao *et al*. and Ponglowhapan *et al*. is that this study uses real time PCR techniques with fresh frozen tissue to quantify mRNA expression levels, whilst the former two studies used in situ hybridisation techniques with formalin fixed, paraffin-embedded tissue. It is known that degradation of target nucleic

acids occurs during formalin fixation and paraffin-embedding procedures (Goelz *et al.*, 1985; Rupp *et al.*, 1988), and that artificial mutations can occur (Williams *et al.*, 1999), which would affect analysis (Mc Sherry *et al.*, 2007).

The changes in expression of mRNA for LH- and GnRH- receptors observed in this study could support a role for altered expression of GnRH/LH receptors in the development of acquired urinary incontinence in the neutered bitch. It is worthy of note that increases in LH and GnRH receptor mRNA expression also occurred in the bladders of neutered male canines, however, which do not typically become incontinent. The magnitude of the increase in expression for both receptor mRNA species, however, was smaller than in the females in association with neutering and the final levels of expression were only marginally above those of entire female canines which also rarely become incontinent, thus it is possible that there is a threshold effect. The case for a threshold level of LH- and GnRH- receptor expression, above which urinary incontinence is more likely to develop is supported by the fact that the three animals included in the study that were known to be suffering from acquired urinary incontinence (all neutered females) had the highest observed LH- and GnRH receptor mRNA expression levels.

The exact role of the LH receptor in the urinary bladder has not yet been described. It has previously been reported that plasma concentrations of LH increase in both post menopausal women and in neutered canines (Overlie *et al.*, 1999; Reichler *et al.*, 2004), but that LH in both populations is still released in a pulsatile manner. It is also known that these two subsets of their respective populations are more prone to developing urinary incontinence. This present study has shown that neutering causes an increase in mRNA expression levels for the LH receptor in the canine urinary bladder and that these expression levels are highest in bitches known to be suffering from acquired urinary incontinence. It has also been shown that maximal bladder contractility is decreased in neutered animals and is lowest in bitches suffering from acquired urinary incontinence and that there is a direct inverse correlation between bladder contractility and mRNA expression levels for LH. It therefore follows that the increase in mRNA for the LH receptor may be implicated in the decrease in contractility of the detrusor muscle, either directly or via interactions with another mechanism, possibly involving the muscarinic receptor effector pathway, the primary pathway involved in bladder contraction (Chess-Williams, 2002).

The exact role of the GnRH receptor within the urinary bladder has not been characterised in any species. However, based on a recent study that reported increases in bladder

threshold volume following administration of GnRH analogues to neutered continent bitches it has been hypothesized that it may have a role in determining bladder function (Reichler *et al.*, 2006a). This could occur if GnRH receptor expression modulated the bladders sensitivity to muscarinic stimulation to affect the bladder threshold volume, possibly via the sympathetic nervous system where GnRH has been shown to act as a co-transmitter in cholinergic nerve terminals (Jan *et al.*, 1983). As a direct receptor-mediated effect of GnRH on smooth muscle cells of the gastro-intestinal tract has also been demonstrated in rats (Chen *et al.*, 2004), it is plausible that GnRH could exert direct effects on the detrusor smooth muscle in canines. This hypothesis is supported by the data contained within this study where a decrease in *in vitro* detrusor muscle sensitivity to muscarinic stimulation was seen in neutered canines when GnRH receptor mRNA expression levels were found to be high. Thus, the increase in GnRH- receptor expression could be associated with a decreased sensitivity of the detrusor which would allow further relaxation of the bladder and increased capacity.

While two other studies (Ponglowhapan *et al.*, 2007b; Welle *et al.*, 2006) have reported FSH mRNA expression in the canine bladder the current study does not document significant levels of mRNA expression. The reason for this difference could be methodological, as Ponglowhapan *et al* (Ponglowhapan *et al.*, 2007b) used *in situ* hybridization whilst this study used real time PCR techniques. Alternatively it could be related to differences in the FSH mRNA sequences used. This present study used the same primer and probe sequences published by Welle *et al* (Welle *et al.*, 2006) and unsurprisingly the results are more similar to those of Welle *et al* than Ponglowhapan *et al*. To verify the results of the present study the presence of FSH mRNA in both the ovary and skin was examined as positive controls (Minegishi *et al.*, 1997; Welle *et al.*, 2006). The results of this present study supported the results of Welle *et al* but the lack of expression in the ovary which is known to be sensitive to FSH action would suggest that this primer probe combination is picking up a non reproductive FSH receptor isoform. Indeed, FSH is known to express multiple isoforms (Zambrano *et al.*, 1999) therefore it is a possibility that the primers and probes used in this study and that of Welle *et al* (Welle *et al.*, 2006) were designed against part of the mRNA sequence of an FSH receptor isoform that is expressed in skin but not in the bladder. Thus, at this point, it is not possible to make any definitive conclusions with regard to the effects of neutering on FSH receptor mRNA expression within the bladder wall.

In conclusion this study has shown that neutering a canine, regardless of gender, is associated with an increase in the levels of mRNA for LH- and GnRH- receptors in the

canine urinary bladder, and that female animals, regardless of gonadal status have higher LH/GnRH receptor mRNA expression levels than males. A further novel observation shown by this study is the positive correlation between increased mRNA expression for LH- and GnRH- receptors and the negative correlation between expression of these receptors and contractility of the detrusor to muscarinic stimulation *in vitro*. It is hypothesized that the changes in mRNA expression for both GnRH- and LH- receptor may have a role to play in the development of post neutering acquired urinary incontinence and this hypothesis is supported by data from a limited number of neutered female canines identified as suffering from acquired urinary incontinence which had the highest levels of LH- and GnRH- receptor mRNA expression and the lowest contractility to muscarinic stimulation of all the animals studied.

10 Thesis Overview

Canine acquired urinary incontinence is known to be a widespread problem in a variety of countries around the world, with up to 20% of all neutered bitches affected, compared to less than 1% of entire bitches and male canines (Arnold *et al.*, 1989). The condition can be severely debilitating and can lead to a number of further problems and complications including urine scalding, skin infections, secondary bacterial cystitis and even sepsis and death (Hotston Moore, 2001). The development of acquired urinary incontinence in a bitch prompts welfare considerations, and presents a financial burden to the owner who, in addition, is often required to make lifestyle changes to accommodate their pet's needs. This can all lead to the difficult and emotionally traumatic decision to have the affected animal euthanized.

Although acquired urinary incontinence in the canine has been recognized for a number of years, the exact pathophysiology of the condition remains unknown. Furthermore, regardless of advances in diagnostic and surgical techniques and the development of new pharmacological therapies, it is still impossible to affect a long term cure for this disease due to either diminishing of the surgical effect or a refractory response to pharmacological therapy seen over time (Angioletti *et al.*, 2004; Arnold, 1997b; Holt, 1990; Hotston Moore, 2001; Janszen *et al.*, 1997; Mandigers *et al.*, 2001; Reichler *et al.*, 2006).

It has been shown that neutering a bitch is directly linked to the development of acquired urinary incontinence, and it has also been demonstrated that it is not mechanical damage of the urinary tract during this procedure that leads to the development of the disease (Gregory, 1994). Further studies have shown a positive correlation between increasing normal adult body weight of a bitch and the development of acquired urinary incontinence, as well as between obesity and the development and severity of the disease (Arnold *et al.*, 1989; Holt *et al.*, 1993); this latter relationship is likely to become more important as the number of obese pets in our society rises. There is also a positive correlation between tail docking and the disease, likely due to damage of the nerves that leave the sacral spinal cord during the procedure (Holt *et al.*, 1993), although this is likely to play a decreasing role now that tail docking for cosmetic reasons has been banned in the United Kingdom and elsewhere.

It has been reported that neutering a bitch leads to a decrease in the resting urethral tone (Gregory *et al.*, 1996; Holt, 1988; Reichler *et al.*, 2004; Rosin *et al.*, 1981). This decrease

in tone can in turn lead to clinical signs of urinary incontinence as the urethra is no longer able to overcome any increases in pressure within the bladder, such as when the animal lies down or sneezes (Gregory *et al.*, 1996; Rosin *et al.*, 1981). Although there is no doubt that this decrease in urethral tone has a role to play in the development of acquired urinary incontinence in the bitch, it is not a defining characteristic of the disease, as increasing the urethral tone will not restore continence in all bitches and some bitches will regain continence without an increase in their urethral closure pressure (Reichler *et al.*, 2006).

As the urethra may, therefore, not be wholly responsible for the development of acquired urinary incontinence in the bitch I hypothesized that other factors may be involved. It has long been recognized that acquired urinary incontinence in the bitch is linked to gonadectomy and the resultant changes in reproductive hormones that this entails (Holt *et al.*, 1993; Thrusfield, 1985). This is similar to our understanding in those women who develop urinary incontinence post menopause (Thom *et al.*, 1998) where the hormone status is similar to that of a neutered bitch. As a known cause of urinary incontinence in post-menopausal women is the bladder and the structural and functional changes that can take place within it (Abrams *et al.*, 2002; Andersson, 2003; Holroyd-Leduc *et al.*, 2004b; Thom *et al.*, 1998), I hypothesized that changes at the level of the bladder, in terms of bladder contractility, structure and receptor compliment, may also have a role to play in the development of acquired urinary incontinence in the bitch.

Although there have been no reported urodynamic studies in bitches to determine the exact role of the bladder in the overt clinical signs of urinary incontinence, anecdotal evidence exists to support a role for this organ. Whilst the most common presentation of acquired urinary incontinence in the bitch is that she unconsciously loses urine whilst asleep (Arnold, 1997a), a proportion of owners report that their animal gets very agitated immediately before the incontinent episode and may be seen to try and get to their usual toileting place, often getting ‘caught short’ before they make it. This is similar in presentation to women who suffer from urge incontinence who find the sudden onset of the urge to urinate discomforting and who may not reach a suitable place to urinate before unconscious leakage of urine occurs (Holroyd-Leduc *et al.*, 2004a) and therefore suggests that the bladder may have a role to play in acquired urinary incontinence in a number of bitches.

The aim of this thesis was therefore to investigate whether functional, structural and molecular changes occur within the bladder of a canine post neutering that may lead female canines to develop urinary incontinence. This was achieved via a number of

methods including *in vitro* tissue bath experiments to determine if neutering altered the maximal contractile response and sensitivity of isolated strips of canine detrusor muscle in response to both muscarinic and electrical field stimulation in both male and female canines. Males were included as they undergo similar hormonal changes post neutering but few develop urinary incontinence. The studies also included a small number of neutered female canines known to be suffering from acquired urinary incontinence. Studies then explored the mechanisms that might underlie the changes seen in these initial experiments. This included the pharmacological characterization of the muscarinic mediated response of the tissue strips, as well as considering the effects of short-term incubation of bladder smooth muscle strips with reproductive hormones. Further studies included morphometric analysis of the canine urinary bladder to determine changes in percentage collagen within the bladder post-neutering; whilst the mRNA expression levels for the muscarinic receptors and for hormonal receptors within the bladder wall were also investigated.

The initial studies into the effects of neutering on the *in vitro* responses of canine detrusor muscle strips to carbachol and electrical stimulation showed a significant effect of neutering on both maximal response and sensitivity of the strips to muscarinic stimulation, and the maximal contractile response seen to electrical field stimulation. The studies showed that neutering a canine of either gender resulted in a lower maximal contractility and sensitivity of the detrusor muscle strips to both carbachol and electrical field stimulation. It was also shown that the few animals known to suffering from acquired urinary incontinence at the time of the study had the lowest maximal contractile response of all the animals studied and that there was a positive correlation between the maximal contractile response to carbachol and electrical field stimulation in all animals. This later correlation was to be expected as both carbachol and electrical field stimulation are known to act via the muscarinic pathway (D'Agostino *et al.*, 1989). Although the decrease in response is seen in both male and female canines it is known that female canines are at significantly greater risk of developing urinary incontinence. This is thought to be due to the differences in the anatomy of the lower urinary tract in males compared to female canines, as male canines have a significantly longer urethra which passes through the penile structures leading to a higher urethral closure pressure which is exerted over a much greater length (Dyce *et al.*, 2002) . This suggests that decreased responses seen in neutered animals may be involved in the development of acquired urinary incontinence in the bitch, and suggest a functional role for the bladder in the disease process.

It is probable that the decreased responses seen *in vitro* are mimicked *in vivo*, in which case they would be expected to be responsible for lower magnitude contractions of the bladder which may be insufficient to empty the bladder fully leading to small residual volumes of urine post voiding and the propensity to develop cystitis. This change in contractility post steroid loss is similar to that reported in a subset of women suffering from urinary incontinence due to impaired contractility of the bladder (Elbadawi *et al.*, 1993a; Resnick *et al.*, 1987). Although many women who suffer from urge incontinence may have impaired contractility of the bladder (Resnick *et al.*, 1987), a proportion of these women also suffer from detrusor overactivity which has been reported to cause an increase in response to carbachol stimulation *in vitro* (Stevens *et al.*, 2007). As the canines known to be suffering from acquired urinary incontinence in this present study had decreased responsiveness it may be that the post-neutering canine bladder is more akin to that of women suffering from impaired contractility of the bladder than those with detrusor overactivity although, more animals need to be studied before firm conclusions can be drawn.

Although both carbachol and electrical field stimulation are known to act via the muscarinic pathway (D'Agostino *et al.*, 1989; Rang *et al.*, 2007) it has been shown in other species that part of the response to electrical field stimulation can be mediated via the non-adrenergic, non-cholinergic system (Andersson *et al.*, 2004). Further studies in canines were therefore conducted to look at the non-adrenergic, non-cholinergic system and to determine if this was altered in neutered compared to entire canines. These studies demonstrated that there is a non-adrenergic, non-cholinergic mediated portion to the contractile response in the canine, and that the proportion it contributes lies between that of the rat and human (Andersson *et al.*, 2004). The proportion it contributes in the canine does not alter significantly between neutered and entire animals; however, there is a trend towards neutered animals having a greater non-adrenergic, non-cholinergic component of contraction than their entire counterparts. This trend is similar to that seen in human patients suffering from bladder outlet obstruction and detrusor overactivity (Andersson *et al.*, 2004b) thus supporting the original hypothesis that stated that gonadectomised animals would have a significantly higher proportion of the contractile response mediated by the non-adrenergic, non-cholinergic system. Although this trend may have a minor role to play in the decreased contractility of the bladder seen in neutered compared to entire canines it is probable that alterations in the muscarinic receptor or receptor/effector pathway have the greatest influence on bladder contractility post-gonadectomy.

As all muscarinic receptor subtypes are expressed in the bladder wall and as it has been reported that the functionally important subtype can be altered in disease states (Chess-Williams, 2002) I then undertook both pharmacological characterization studies to determine the muscarinic receptors responsible for the *in vitro* contraction to carbachol and molecular studies to determine the amount of expression of the different muscarinic receptor subtype mRNAs within the canine urinary bladder wall. Pharmacological characterization of the muscarinic receptor subtypes using pirenzepine, methoctramine and 4-DAMP, selective antagonists of the M_1, M_2 and M_3 receptors respectively (Caulfield *et al.*, 1998), demonstrated that although the M_3 receptor is mainly responsible for bladder contraction in entire animals, the M_2 receptor appears to dominate in neutered animals. As this is the first study to investigate effects of gonadectomy on the muscarinic subtypes mediating bladder contraction it is not known if this finding is unique to the canine or if it is repeated in other species. It has been reported by one group studying micturition in the obstructed rat bladder that there is a shift from M_3 to M_2 in this disease state (Braverman *et al.*, 1999; Braverman *et al.*, 2003; Braverman *et al.*, 2006), however, another group studying a similar model could find no evidence of M_2 receptor involvement (Krichevsky *et al.*, 1999), possibly due to differences in experimental techniques and animal numbers. This is similar to the picture in humans where one group claimed a role for the M_2 receptor in patients suffering from neurogenic overactivity of the bladder (Pontari *et al.*, 2004), whilst another group concluded that only the M_3 receptor was responsible (Stevens *et al.*, 2007). These conflicting results may be due to differences in experimental design, low patient numbers and individual variation in patients / animals, as well as in disease state. It is therefore plausible that the shift towards the M_2 receptor seen in the neutered canines in this study may be involved in the decreased bladder contraction seen *in vitro* and in the development of acquired urinary incontinence in the canine.

It has been demonstrated that the M_2 receptor does have a role to play in bladder contraction *in vitro* in some species as shown by the decreased responsiveness of detrusor muscle strips to muscarinic stimulation seen in M_2 knock-out mice (Stengel *et al.*, 2000). This is further supported by a rodent study that identified both the M_1 and M_2 receptors as being involved in bladder contraction (Frazier *et al.*, 2007). This work is complemented by a study in anaesthetized rats that has demonstrated an *in vivo* role for the M_2 receptor in mediating bladder contraction (Hegde *et al.*, 1997). As the contractions mediated by the M_2 receptor are postulated to be weaker than those mediated by the M_3 receptor, at least in pigs (Yamanishi *et al.*, 2000), it is possible that the shift from the M_2 receptor to the M_3 receptor as seen in neutered canines in this present study is responsible for the decreased contractile function seen.

Although a role for the M_2 receptor in mediated bladder contraction has been demonstrated the exact mechanism by which it affects this response has not been reported. It has been postulated that the M_2 receptors may mediate smooth muscle tone under conditions of high sympathetic activity (Eglen *et al.*, 1994) whereby the activation of the M_2 receptors effectively switches off the sympathetic inhibitory mechanisms mediated by the β-adrenoceptors leading to improved contraction and emptying of the bladder. This is hypothesized to occur via activation of a GTP binding protein associated with inhibition of adenylyl cyclase (Braverman *et al.*, 1999; Hegde *et al.*, 1997). Although specific muscarinic receptor subtypes appears to preferentially couple to particular G proteins there does now appear to be considerable promiscuity in this coupling mechanism (Tucek *et al.*, 2002). This means that depending on the particular cell type and the biochemical state of the cell a single subtype of receptor may be able to couple with several different types of G protein, meaning that a single receptor could mediate a number of cellular signaling pathways to effect differing responses (Tucek *et al.*, 2002; Wang *et al.*, 1995). Traditionally the M_2 receptor has been thought to couple with the G_i class of GTP binding proteins, however it has been shown, in the human bladder, that they can also bind to the G_q class of proteins (Wang *et al.*, 1995), the same class of G proteins that the M_3 receptor classically binds to (Rang *et al.*, 2007). It therefore follows that differential coupling of the M_2 receptor to different G proteins may alter its effect on bladder tissue and may allow it to assume the role of primary muscarinic receptor responsible for bladder contraction in certain disease states, potentially including acquired urinary incontinence in the canine.

Although, as previously stated, the M_3 receptor is considered the primary receptor responsible for bladder contraction in healthy individuals it is the M_2 receptor which is the most numerous muscarinic receptor within the bladder of all species so far studied (Chess-Williams, 2002). The results for mRNA expression levels obtained in canines in this study are in agreement with this, although the ratio of M2:M3 receptors is slightly lower than that of other species studied at 1.5:1, compared to 3:1 in the human and 9:1 in the rat (Chess-Williams, 2002). These differences may reflect inter-species or subject differences or variations in protocol and methodology, however, the functional role of the M_2 and M_3 receptors in rats and humans appear to be similar and mimic that found for healthy entire canines in this study.

The mRNA expression levels for the M_1, M_2 and M_3 receptors, regardless of gender, were shown to be increased in neutered compared to entire canines in this study. The expression levels for the M_2 receptor were also shown to be highest in neutered female canines known to be suffering from acquired urinary incontinence. Further analysis showed a significant

negative correlation between expression levels of mRNA for the muscarinic receptors and maximal contraction of the bladder *in vitro* to muscarinic stimulation. Taken with the results from the pharmacological characterization of the muscarinic receptors which showed a shift towards the M_2 receptor in mediating bladder contraction in neutered animals these results are significant. It has been shown in rats suffering from denervation of the bladder that the expression of the M_2 receptor is enhanced in certain disease states, and this model also demonstrated the significant contribution of the M_2 receptor in mediating bladder contraction (Braverman *et al.*, 1998b). The increased amount of mRNA for the M_2 receptor in the canine may therefore be involved in the increased role of the receptor in the function of the bladder and, if mimicked by changes in receptor protein, may provide the basis for a potential therapeutic target for treating acquired urinary incontinence in the bitch.

Considering the finding of the pharmacological characterization study showing the shift from M_3 to M_2 receptor in neutered canines it may have been predicted that the expression levels for the M_3 receptor would decrease in the neutered compared to entire canines, however the results of this study show the opposite to occur. This seemingly contradictory set of data may be due to a number of factors, including the possibility that the increased M_3 receptor mRNA is not accompanied by an increase in the M_3 receptor protein, possibly due to alterations in translation of the gene. It has been shown in a recent study in rats that the mRNA expression and protein levels for this specific receptor may not be correlated (Braverman *et al.*, 2006), therefore further studies to investigate protein levels of the muscarinic receptors in the canine are warranted. It is also plausible the increased expression of the M_3 receptor mRNA seen in neutered canines is a compensatory response to the decreased detrusor contractility brought about by an as yet unidentified mechanism that may involve the M_3 receptor itself.

As the functional role of the M_1 receptor in the canine bladder cannot be established from the studies so far conducted the significance of the increased expression levels of mRNA in neutered canines cannot be ascertained. It is known that the M_1 receptor is usually found on pre-synaptic nerve terminals where it is thought to facilitate neurotransmitter release (Braverman *et al.*, 1998a; Somogyi *et al.*, 1999; Somogyi *et al.*, 1994). If this is the case in the canine it is possible that the M_1 receptor is up-regulated in neutered canines to facilitate transmitter release and thereby increase the contractile force of the bladder, however the contribution of this role, if it exists, is likely to be small.

Although it has been demonstrated that the primary pathway responsible for bladder contraction is the muscarinic pathway, it is possible that changes to other receptors and pathways may also modulate bladder contraction, therefore may be involved in the decreased contractility of the canine detrusor *in vitro* seen in neutered animals. It has been a long held hypothesis that changes in the sex hormones of a bitch post neutering lead her to develop acquired urinary incontinence (Arnold *et al.*, 1989; Stenberg *et al.*, 1995; Thom *et al.*, 1998; Thrusfield, 1985), and a similar hypothesis, involving changes in gonadal steroids, has been considered in the development of urinary incontinence in post-menopausal women (Stenberg *et al.*, 1995; Thom *et al.*, 1998). Post-menopause and after neutering there is a significant decrease in circulating endogenous oestrogen and this has been hypothesised to be a causative factor in the development of urinary incontinence in both the bitch (Finco *et al.*, 1974) and woman (Freedman, 2002). This decrease in circulating oestrogen following menopause / gonadectomy is also known to interrupt the feedback mechanisms that act at the level of the hypothalamus and pituitary gland to regulate the secretion of gonadotrophin hormones leading to an increase in the secretion of both FSH and LH (Burger, 1996; Olson *et al.*, 1992; Reichler *et al.*, 2004). It has been hypothesized that this increase in gonadotrophins may be partially responsible for the development of urinary incontinence in both the canine (Reichler *et al.*, 2004), and woman (Tao *et al.*, 1998), and studies have demonstrated the presence of receptors for the gonadotrophins in the urinary bladder (Ponglowhapan *et al.*, 2007a; Reichler *et al.*, 2007; Tao *et al.*, 1998). Further support for this indirect effect of steroid removal is provided by a recent study in neutered canines suffering from acquired urinary incontinence which reported that administration of GnRH analogues, such as to decrease serum LH and FSH levels, aided continence (Reichler *et al.*, 2006). Part of this thesis therefore looked at the effect of neutering on the expression levels of mRNA for the FSH, LH and GnRH receptors in the canine urinary bladder. Although another study has reported the FSH receptor in the canine bladder (Ponglowhapan *et al.*, 2007b) it was not consistently present at detectable levels in this study. The levels of both GnRH and LH were detectable however, and expression levels of mRNA for both of these receptors, relative to β-actin, were increased in neutered canines of either gender in this study. This is in contrast to other studies in the canine that showed either no change in these receptors (Reichler *et al.*, 2005), or a decrease in their expression in neutered bitches (Ponglowhapan *et al.*, 2007a). The differences in results between studies may reflect a number of factors including small sample size, time from neutering to sampling, differing stages of the oestrus cycle in the entire bitches included as controls, as well as technique used. This present study used real time PCR techniques with fresh frozen tissue, however the other studies have used in situ

hybridization techniques with formalin fixed, paraffin-embedded tissues which is known to allow degradation of target nucleic acids (Goelz *et al.,* 1985; Rupp *et al.,* 1988) and artificial mutations to occur (Williams *et al.,* 1999), affecting analysis (Mc Sherry *et al.,* 2007).

It is possible that the changes in expression of mRNA for both LH and GnRH receptors observed in this present study may be involved in the decreased bladder contractility seen *in vitro* as further analysis showed a negative correlation between increasing mRNA expression levels and decreasing contractility. This hypothesis is supported by the data from the neutered female canines known to be suffering from acquired urinary incontinence that not only had the lowest contractility but also the highest expression levels for both the LH and GnRH receptors. To investigate this, further studies were conducted to determine the effects of short term *in vitro* incubation with LH, FSH, GnRH and oestrogen on the responses of strips of detrusor muscle to muscarinic stimulation. These studies could find no significant effect of these hormones on either maximal contractile response or sensitivity of the detrusor to muscarinic stimulation. This may be a reflection of the *in vivo* system, suggesting that although there may be changes in the mRNA expression levels for some of these hormone receptors within the bladder these do not produce functional changes. It is also conceivable, however, that functional changes within the bladder take considerably longer to develop than the constraints of this study allowed, as demonstrated by the fact that the responses of bitches suffering from acquired urinary incontinence to hormonal treatment can take weeks to fully develop (Angioletti *et al.*, 2004; Reichler *et al.*, 2003). Furthermore, changes in responsiveness may be secondary to alterations in bladder structure or receptor number brought about by chronic hormone exposure over a number of weeks or months, thus indicating that prolonged exposure *in vivo* would be required before functional changes are seen *in vitro*. This is supported by reports that chronic changes in oestrogen concentrations can cause structural changes to the urinary bladder in rodents which are thought to contribute to alterations in bladder function (Fleischmann *et al.,* 2002).

The structural changes seen in the urinary bladder in rodents (Fleischmann *et al.*, 2002) are also seen in women where they are thought to contribute to urinary incontinence. In post-menopausal women suffering from both impaired contractility of the bladder and idiopathic detrusor instability there is an increased collagen to smooth muscle ratio (Elbadawi *et al.*, 1993a; Elbadawi *et al.*, 1993b) which is hypothesized to alter electrical conductivity within the smooth muscle bundles of the bladder and therefore alter the contractility of the muscle (Charlton *et al.,* 1999; Chen *et al.,* 2002). As it is known that

women suffering from impaired contractility of the bladder can have decreased responses to muscarinic stimulation *in vitro*, as reported in this canine study, it was hypothesized that similar changes may occur in the bladder of neutered canines. This was proven to be the case in the female canines studied, however, this hypothesis did not hold true for neutered male canines that had the same collagen percentage as their entire counterparts. As this is the only reported study looking at collagen percentage within the bladder of gonadectomised males of any species, it is not known if the changes seen are particular to the canine or if they are representative of the male gender as a whole. Regardless of this, the changes in percentage collagen seen in this study cannot be wholly not responsible for the decreased contractility of the canine bladder seen *in vitro*, as the decreased contractility occurs in neutered canines of both genders, whilst the increase in percentage collagen is unique to the neutered female.

This observed increase in collagen within the bladder wall of the neutered female canines appeared to occur both within and between the muscle bundles that make up the detrusor muscle. This has the potential to decrease the contractility and elasticity of the detrusor and / or affect bladder compliance, in turn affecting the bladder's ability to relax and expand to store urine, a feature of the bladder which has been linked to urinary retention and incontinence in the human (Chen *et al.*, 2002). An increase in percentage collagen has also been reported in the wall of the urinary bladder of women with detrusor instability, where it is associated with an altered sensory threshold for cholinergic stimulation of the bladder (Charlton et al., 1999). It is also hypothesized that intramuscular collagen deposition will decrease conduction of action potentials throughout the muscle fascicle (Fleischmann et al., 2002), and thus could have a negative effect on the ability of the bladder to respond to stimulation as a single functional unit. The possibility that the observed increases in collagen relative to smooth muscle tissue may predispose a neutered bitch to develop acquired urinary incontinence is supported by the data from those animals known to be suffering from acquired urinary incontinence that had some of the highest percentage collagen but the lowest responses to muscarinic and electrical field stimulation of all animals tested. It is therefore feasible that although the increase in percentage collagen cannot be wholly responsible for the decreased contractile function of the bladder in neutered canines, it may still be involved, possibly in a synergistic manner, alongside other mechanisms such as changes in the muscarinic or purinergic pathways or sex hormone receptors.

The data presented in this thesis together with previously reported investigations of urethral function by other groups suggests that acquired urinary incontinence in the bitch

has a multifactorial pathogenesis, with changes occurring in both the urethra and the bladder which may contribute to the disease development. Among the changes seen within the bladder of neutered canines of both genders are a decreased maximal contractility and sensitivity of the detrusor muscle to muscarinic stimulation *in vitro*, a functional shift from the M_3 to the M_2 receptor mediating this contraction, and an increase in the expression levels of mRNA for the M_1, M_2 and M_3 receptors. Other changes demonstrated include an increase in the expression levels of mRNA for the LH and GnRH receptors, although the functional significance of these are unknown as short term incubation of detrusor muscle strips with these hormones, as well as FSH and oestrogen, produced no change in contractility *in vitro*. Finally, increased percentage collagen within the wall of the urinary bladder of neutered bitches has been reported and has been inversely correlated with the decreased bladder contractility recorded and this relationship has been shown to be especially prominent in those animals known to be suffering from acquired urinary incontinence.

Many of these changes are similar to those of women known to be suffering from urinary incontinence due to various conditions of the bladder such as the decreased functional responses to muscarinic and electrical field stimulation seen in neutered canines *in vitro* which are akin to those seen in women suffering from impaired contractility of the bladder. The change towards the M_2 receptor in neutered canines is similar to that of women with detrusor overactivity and the increase in collagen in neutered bitches is similar to that of women suffering from urinary incontinence due to idiopathic detrusor instability with or without impaired contractility of the bladder. In contrast, the increased expression level of mRNA for the LH receptor in neutered canines is the opposite of that reported by a study in women which found a decrease in LH mRNA levels in post- compared to pre-menopausal women; although the link between these changes and urinary incontinence in either species is not clear. It is therefore plausible to say that the bladder of the neutered bitch and the post menopausal woman share a number of common characteristics, along with the propensity to develop urinary incontinence, and therefore further investigation into the bladder of the bitch post neutering is warranted both to identify possible therapeutic targets for treatment of acquired urinary incontinence in the bitch and to confirm the gonadectomised canine is a suitable model to study aspects of post-menopausal urinary incontinence in the woman.

There have been many studies looking at the dynamic function of the bladder in the woman however none have been reported in the bitch. Due to the constraints of communication between researchers and canines it is not possible to known if a bitch

suffers from similar symptoms to that of incontinent women, such as urgency and partial involuntary bladder contractions. A long term *in vivo* study observing normal micturition and bladder parameters in both healthy bitches before and after neutering, as well as in animals known to be suffering from acquired urinary incontinence, would therefore enhance our understanding of the processes involved in micturition in normal and incontinent animals. This would also allow us to evaluate the time scale involved in the development of bladder and urethral changes post neutering. This could be achieved via the use of telemetry with a transmitter-catheter device placed in bitches a number of months prior to neutering, and maintained during and after gonadectomy for a further number of months / years. Using this protocol it would also be possible to hormonally treat a number of animals using both oestrogen and GnRH analogues both before and after gonadectomy to monitor changes in bladder function brought about by chronic hormone therapy. Ideally bladder tissue from all of the animals involved would also be harvested before and after gonadectomy for both *in vitro* functional studies and molecular characterisation. This would also potentially allow age and breed matched animals to be included in the studies, factors that were not possible to control in this thesis due to the source of tissues obtained. Although statistical analysis throughout this thesis did not demonstrate an effect of age or weight of the animals, the tissue obtained came from many varied and mixed breeds; it would therefore be beneficial to have matched groups to negate as much variation as possible.

Even though this thesis has demonstrated a number of changes post-neutering in the bladder of a bitch that may be involved in the development of acquired urinary incontinence, the low number of animals positively identified as suffering from the disease has made accurate and robust statistical analysis of this group impossible. Any further studies should therefore look to involve a greater number of such animals, a minimum of 6 animals is required to allow statistical analysis and ideally 10 or more animals to make the statistical analysis robust. One possible way of obtaining tissue from this group of animals would be to take small (2cm by 1cm) full-thickness biopsies from the bladder dome at the time of other abdominal or bladder surgery. Although this is unlikely to yield enough tissue for extensive tissue bath experiments it would allow molecular studies into mRNA expression levels, and possibly into protein expression levels, as well as yield 4-8 strips of detrusor muscle for *in vitro* functional studies.

The *in vitro* functional studies so far conducted investigating the muscarinic receptor subtype responsible for bladder contractions in the canine have yielded potentially significant results and therefore warrant further investigation. As well as including more

animals in this study, especially those known to be suffering from acquired urinary incontinence, additional specific muscarinic antagonists should be utilised. There is a degree of overlap between the specificity and affinity of muscarinic antagonists, therefore studying the effects of a greater number of these, especially those that are specific for the M_2 and M_3 receptor subtypes, such as gallamine and darifenacin, would make the study more robust.

Along with the *in vitro* functional muscarinic receptor studies the *in vivo* effects of various antimuscarinic compounds such as tolterodine and propantholine bromide, which may make suitable future treatments for acquired urinary incontinence in the canine, should be tested on entire and neutered canines, as well as those known to be suffering from acquired urinary incontinence. This would best be achieved by the use telemetry to monitor bladder responses as well as cardiac function, as many antimuscarinic agents may also affect cardiac function. Close monitoring of other body systems and parameters such as tear production, salivation and gastric motility would also be advisable to monitor for adverse effects of the drugs on other body functions. Telemetry should be started a number of weeks before the agents are administered and continued throughout the treatment period. As with the study into the *in vivo* functional effects of gonadectomy, bladder tissue could be harvested at various time points in this study for *in vitro* pharmacological characterisation studies, as well as for molecular studies to monitor any changes in the expression levels of mRNA and protein for the muscarinic receptors.

The mRNA studies undertaken for this thesis have shown significant differences in the expression levels of mRNA for some of the muscarinic receptors, as well as for GnRH and LH. As these changes in receptor levels have also been correlated with the decreased contractility of the neutered canine bladder *in vitro* further investigation into the exact location of the receptors, their protein expression levels and whether they are co-expressed is advised. Investigation into the receptors so far studied, as well as FSH and oestrogen (α and β) has the potential to provide important insights into the pathophysiology of the bladder and its role in the development of acquired urinary incontinence and may also provide therapeutic targets for effective treatment of this debilitating disease.

Although the molecular studies investigating the expression levels of mRNA for the muscarinic receptors have yielded potentially significant data, the expression levels for the receptor proteins remain unknown. Work on this area was initiated during the course of this thesis by The University of Glasgow's Pathology Department, using commercially available mouse and rabbit raised antibodies to the individual muscarinic receptors in

immunocytochemistry studies; however the proteins were not detected. This may be due either the experimental conditions or antibodies used. The antibodies and techniques were proven to work in mouse control tissue, therefore operator or technique errors are unlikely. To maximise the chance of the antibodies binding the experiments were also conducted under a variety of standard and non-standard conditions. As there is only thought to be approximately 90% homology between the sequences for the muscarinic receptor subtypes in the canine and the mouse and rabbit, it may be that the antibodies used were not specific enough to bind to the canine receptors. To investigate the protein levels of the muscarinic receptor subtypes in the canine bladder it would therefore be necessary to develop canine specific antibodies, however this was beyond the budget capabilities and time limitations of this thesis. It may also be possible to employ further techniques to study the protein levels of all the receptors so far mentioned, including western blotting and radioligand binding, although specific antibodies may again need to be custom designed for some of these studies.

A further area for investigation into the role of the bladder in the development of acquired urinary incontinence in the bitch would be to look for the presence and effect of any urothelium derived relaxing factor(s) on the contractility of the detrusor muscle. This factor, the exact nature of which is still unknown, has been identified in both human and pig bladder, and has the potential to alter the contractile function of the bladder in various disease states. One way in which to study the influence of diffusible factors produced by the urothelium on the contraction of the detrusor muscle, in response to both pharmacological and electrical stimulation in the canine would be to use both detrusor and anococcygeus (control) muscle. Smooth muscle strips from both muscles would be mounted in a classical tissue bath and enveloped in a layer of urothelium, thus allowing evaluation of the influence of urothelium-derived relaxing and contracting factors on the enclosed smooth muscle strip. As with all the experiments thus conducted tissue derived from male and female, entire and neutered canines as well as those known to be suffering from acquired urinary incontinence should be used.

In conclusion, the results of this thesis have provided invaluable insights into the role of the canine urinary bladder in the development of acquired urinary incontinence in the bitch, a debilitating and so far incurable condition known to affect up to a fifth of all neutered bitches. This thesis has also established that the bladder of a neutered bitch has many similarities to that of a post-menopausal woman. The studies have demonstrated a decrease in the *in vitro* contractility of the detrusor from neutered canines, and have linked this to both gross structural and molecular level changes. From the information so far

gathered it appears that changes in the muscarinic receptor pathway have a potentially significant role to play in the development of acquired urinary incontinence and, as such, may prove to yield future therapeutic targets for treatment of this serious disease, potentially by drugs such as propantheline bromide and tolterodine. Further investigation is now warranted into both the muscarinic and hormonal receptors of the bladder, as well as into further novel mechanisms such as urothelium derived relaxant factors. These further studies would benefit from concurrent *in vivo* functional investigations to help aid our understanding of the pathophysiology and time-course of acquired urinary incontinence in the bitch, as well as the inclusion of an increased number of bitches known to be suffering from acquired urinary incontinence in all parts of the study. Finally, this thesis verifies that the urinary bladder has a role to play in acquired urinary incontinence in the bitch and suggests areas for further investigation that may provide therapeutic targets for effective treatment of the disease.

Appendix - Publications

Papers

Coit, V, Gibson, I, Evans, N, Dowell, F (2008). Neutering affects urinary bladder function by different mechanisms in male and female dogs. *European Journal of Pharmacology* **584(1):** 153-158.

Coit, V, Gibson, I, Evans, N, Dowell, F (2008). Neutering affects mRNA expression levels for the LH- and GnRH- receptors in the canine urinary bladder. *Theriogenology. In Press.*

Abstracts

Coit, V., Gibson, I., Evans, N. & Dowell, F. Influence of neutering in bitches on *in vitro* contractile responses of the canine urinary bladder to cholinergic and electrical stimulation. Scientific Proceedings of the BSAVA Congress (2006).
(Awarded Prize for best Clinical Review Abstract)

Coit, V., Gibson, I., Evans, N. & Dowell, F. *In vitro* contractile response of canine urinary bladder to muscarinic and electrical stimulation: effects of neutering. British Pharmacological Society, Abstracts 75th Anniversary Meeting, C007 (2006).

Coit, V., Evans, N. & Dowell, F. Influence of neutering on collagen in the canine urinary bladder: evaluation by quantitative morphometric analysis. Scientific Proceedings of LifeSciences2007 Meeting (2007).

Coit, V., Dowell, F. & Evans, N. Effects of sex and neutering on mRNA transcription for M_1 and M_2 receptors in the canine urinary bladder. Proceeding of WSAVA 2007 (2007).

Coit, V., Evans, N. & Dowell, F. Influence of neutering on collagen in the canine urinary bladder: evaluation by quantitative morphometric analysis. Proceedings of WSAVA 2007 (2007).

Coit, V., Dowell, F. & Evans, N. M_3 receptor mRNA expression in the canine urinary bladder is affected by gender and neutering. British Pharmacology Society, Winter Meeting, Abstracts (2007).

Coit, V., Dowell, F. & Evans, N. Neutering affects mRNA expression for LH- and GnRH-receptors in the canine urinary bladder. Scientific Proceedings of the BSAVA Congress (2008)

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