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Impact of castration on changes in left ventricular diastolic pressure-volume relations induced by chronic adrenergic stimulation in rats.

Bryan Hodson

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Masters of Science in Medicine.

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Abstract

A reduced testosterone concentration characterizes heart failure and independently predicts outcomes. Although testosterone replacement therapy may have non-cardiac-related therapeutic benefits in heart failure, whether reduced testosterone concentrations protect against adverse left ventricular remodelling (LV dilatation) is uncertain. I therefore evaluated whether surgical castration modifies LV dilatation following 6 months of daily injections of the β-adrenergic receptor (AR) agonist, isoproterenol (ISO) (0.015 mg/kg/day) to rats. The extent of LV dilatation and LV systolic chamber dysfunction were determined using both echocardiography and isolated perfused heart procedures. A load-independent measure of LV dilatation was determined from the volume intercept of the LV diastolic pressure-volume (P-V) relationships. As compared to the saline vehicle-treated group, after 6 months of β-AR activation in sham-castrated rats, a marked right shift in the LV diastolic P-V relationship was noted with an increased LV volume intercept at 0 mmHg diastolic pressure (LV V_0 in ml)(ISO=0.38 ± 0.02, Saline vehicle=0.30 ± 0.02, $p<0.05$). However, chronic β-AR activation did not alter LV systolic chamber function either *in vivo* (LV endocardial fractional shortening, echocardiography) or *ex vivo* (LV end systolic elastance). Although castration decreased body weight, castration failed to modify the impact of ISO on the LV diastolic P-V relationships or the LV volume intercept at 0 mmHg diastolic pressure (LV V_0 in ml)(Castration ISO=0.35 ± 0.02, Castration saline vehicle=0.27 ± 0.03, $p<0.05$). In conclusion, castration does not influence the extent of LV dilatation induced by chronic adrenergic activation in an animal model where adverse LV remodelling precedes LV systolic chamber dysfunction. These data lend support for the notion that testosterone replacement therapy in heart failure may not produce adverse effects on the degree of cardiac dilatation.

Declaration

I, Bryan Hodson, declare that the work contained in this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work Contained in this dissertation has not been submitted for any other degree or examination in this university, or any other university.

.....

Bryan Hodson

Signed on ..... day of ....., 2014.

I certify that the studies contained in this thesis have the approval of the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics clearance number is 2010-25-04

.....

Bryan Hodson

Signed on ..... day of ....., 2014.

....

Doctor Frederic Michel (Supervisor) Professor Angela Woodiwiss (Supervisor)

Publication, Conference Proceedings and Presentations

The following publication, and oral and poster presentations are offered in support of this dissertation.

1. Publication in press.
    - Hodson, B., Woodiwiss, A. J., Norton, G. R., & Michel, F. (2014). Impact of Castration on Changes in Left Ventricular Diastolic Pressure-Volume Relations Induced by Chronic Adrenergic Stimulation in Rats. *Journal of cardiovascular pharmacology*. PMID: 24477046
2. Oral presentation at the Physiology Society of Southern Africa, hosted by the Dept of Physiological Sciences of Stellenbosch University in 2012.
    - Title: The impact of castration on β-adrenergic stimulation induced changes in heart function and geometry in male rats.
3. Oral presentation at the 2012 Research-Day, Hosted by the Faculty of Health Sciences, the University of the Witwatersrand.
    - Title: The impact of castration on β-adrenergic stimulation induced changes in heart function and geometry in male rats.
4. Poster presentation at the 2012 18th Biennial Congress of the Southern African Hypertension Society.
    - Title: Gender-Specific Effects of Adrenergic-Induced Adverse Cardiac Remodelling in Spontaneously Hypertensive Rats

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List of abbreviations

°C	Degrees Celsius
µl	Microlitre
ALLHAT	Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial
ANOVA	Analysis of Variance
BEST	Beta-Blocker Evaluation of Survival Trial
BMI	Body Mass Index
BW	Body Weight
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CIBIS II	Cardiac Insufficiency Bisprolol Study
CI	Confidence Interval
CO_2	Carbon Dioxide
D-Df	Diastolic dysfunction
EDD	End Diastolic Diameter
Ees	Systolic Elastance
EF	Ejection Fraction
En	Myocardial Systolic Elastance
ESD	End Systolic Diameter
FIRST	Flolan International Randomized Survival Trial
FSend	Endocardial Fractional Shortening
FSmid	Mid-wall Fractional Shortening
g	Grams
HCl	Hydrochloric Acid
Health ABC	The Health, Aging, and Body Composition Study

HW	Whole Heart Weight
ISO	β-Adrenergic Agonist, Isoproterenol
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LV	Left Ventricular.
V_0	Volume Intercept at 0 mmHg pressure
LVDP	Left Ventricular Developed Pressure
LVED	Left Ventricular End Diastole
LVES	Left Ventricular End Systole
LVM	Left Ventricular Mass
MERIT-HF	Metoprolol Extended-release Randomized Intervention Trial
MESA	Multi-Ethnic Study of Atherosclerosis
mg	Milligrams
$MgSO_4$	Magnesium Sulphate
MHz	Mega-Hertz
MI	Myocardial Infarction
ml	Millilitre
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribo-Nucleic Acid
N	Sample Size
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NYHA	New York Heart Association
O_2	Oxygen
OH	Hydroxide
P Value	Probability value

PBS	Phosphate Buffered Saline
PWT	Posterior Wall Thickness
r	Coefficient of determination
SD	Standard Deviation
S-Df	Systolic Dysfunction
SEM	Standard Error of the Mean
β_2	Beta-2
β-AR	β-Adrenergic Receptor

Preface

Despite impressive advances in the understanding and treatment of heart failure and cardiovascular disease during the last few decades, heart failure and cardiovascular disease remain pervasive in society, and prognosis once diagnosed is still incredibly poor. Thus, further understanding of the contributors to the development of heart failure, and possible identification of additional treatment options, remains of vital importance.

One aspect of cardiovascular disease which is still not fully understood is the progression to dilatated, decompensated heart failure; the haemodynamic and geometric changes which occur in such detrimental changes have been well documented, yet the underlying mechanisms and contributors are not yet fully understood. In that regard, there are gender differences in the form of left ventricular dysfunction developed by men and women, such that men have a greater tendency to develop dilatation in heart failure than women, and a greater degree of left ventricular systolic dysfunction; which is supported by other evidence such as gender differences in cardiac geometry and function in men and women at baseline, such that men have greater indices of dilatation and lower indices of function at rest before the development of any disease state. There are gender differences in the neurohumoral, specifically adrenergic, stimulation of the heart, such that male and female hearts respond differently to adrenergic stimulation and there are differences in the efficacy and outcomes of β-adrenergic receptor blockade in heart failure patients.

As such, primarily in the last decade, there has been an increasing focus on the actions of testosterone due to the identified negative correlation of plasma concentrations of testosterone and worsening heart failure in men. This has led to an increasing focus supplementing these testosterone deficient men with exogenous testosterone in the hope of ameliorating their heart failure. However, there is controversy about the actions of testosterone such as stimulating increased dilatation; which, as yet, has not been confirmed or disproven in a chronic model of pump dysfunction or heart failure controlling for the inability

to follow individuals over the course of a lifetime, control for the severity and timing of pathological events, account for treatment differences or the impact of genetic or environmental factors on the heart, or control for the time between initiation of a pathological event and admission for therapy. In contrast, an animal model of the impact of castration on chronic β-adrenergic stimulation induced changes to cardiac geometry and function can control for all of those factors, and allow for a clear demonstration of the contribution of testosterone to dilatation.

In Chapter 1 of the present dissertation, I provide a review of important scientific literature that describes the gender differences in normal function and geometry in humans, how this difference furthers in the development of congestive heart failure, and how gender differences in known contributors such as neurohumoral stimulation may be responsible. After which, I will argue in favour of performing the present study. In chapter 2 I will discuss the methodology employed. Following which, in chapter 3, I will discuss the results obtained. In chapter 4 of this dissertation, I will discuss these results in the context of the scientific literature described in chapter 1, highlight how these results potentially extend our knowledge of the field, underscore the strengths and limitations of the study, and suggest potential clinical and scientific implications for this body of work.

Chapter 1

Introduction

1.1 Introduction

Heart failure is a progressive clinical syndrome with a multitude of possible causes including coronary artery disease, hypertension, diabetes mellitus, obesity, rheumatic heart disease, genetic abnormalities and infections (Jessup *et al* 2003, Chatterjee and Massie 2007, Remme and Swedeberg 2001, Levy *et al* 2002, McMurray *et al* 2010, Cowie *et al* 2000, Mosterd *et al* 2001). Once diagnosed, the survival time in patients with heart failure is similar to that of some of the worst possible malignancies (Lenfant *et al* 1994, Stewart *et al* 2001, Hobbs *et al* 2004). The management of patients with heart failure is estimated to contribute toward a substantial portion of any country's annual healthcare budget (Jessup and Brozena 2003).

Striking advancements have been made over the past three decades in our understanding of the pathophysiological mechanisms responsible for progressive heart failure. In this regard, distinct gender differences have been noted in these mechanisms. Indeed, men with heart failure typically have a poorer prognosis, a higher prevalence of systolic cardiac dysfunction, and a higher prevalence of cardiac dilatation relative to women (Regitz-Zagrosek and Seeland 2011, Konhilas *et al* 2010, Chin *et al* 1998, Adams *et al* 1999, Tamura *et al* 1999, Deschepper *and Llamas* 2007). Thus, the notion that testosterone has deleterious effects on the heart has evolved. Despite these gender differences, and the possibility that testosterone may have deleterious effects on the heart, recent evidence suggests that testosterone deficiency characterises heart failure in men (Jankowska *et al* 2006, Guder *et al* 2010, Kontoleon *et al* 2003, Pugh *et al* 2003, Wu *et al* 2011) and that testosterone deficiency heralds a poorer prognosis in heart failure (Jankowski *et al* 2006, Guder *et al* 2010, Kontoleon *et al* 2003). Thus, testosterone replacement therapy has become a potential therapeutic target in heart failure (Malkin *et al* 2006, Caminiti *et al* 2009, Malkin *et al* 2010).

However, whether testosterone has beneficial or deleterious effects in heart failure is controversial. In light of the recent clinical trials designed to assess the effects of testosterone replacement therapy in heart failure (Malkin *et al* 2006, Hai-Yun *et al* 2011, Caminiti *et al* 2009), the question of whether testosterone is beneficial or deleterious to the heart is of critical importance. In this regard, the important question is whether testosterone replacement therapy is unsafe in patients with heart failure. Hence, in the present dissertation I performed a study to further contribute toward our understanding of whether testosterone deficiency has beneficial, neutral or deleterious actions in cardiac disease.

To assist the reader in understanding the arguments which led me to perform the current study and my choice of animal model of cardiac pathology, in the present chapter I will first provide an overview of the differences that characterise heart failure with either a reduced or a preserved systolic chamber function. I will subsequently describe the gender differences that exist in the pathophysiological mechanisms responsible for either a reduced or a preserved systolic chamber function. Consequently, I will describe the evidence to support or refute the notion that either testosterone has adverse effects on the structure and function of the heart and highlight the conundrum that currently exists with respect to the role of sex-steroid effects on the heart. I will subsequently lead the reader through the evidence to suggest that testosterone replacement therapy may benefit patients with heart failure and consequently summarise my arguments to suggest that further evidence is required to determine whether testosterone replacement therapy is safe in patients with heart failure.

1.2 Cardiac dysfunction in heart failure

Despite the diverse causes of the clinical syndrome, heart failure, from a pathophysiological perspective it has more recently been considered as being either

associated with a reduced or preserved ejection fraction (EF), where EF is a preload-independent measure of systolic chamber function. At a more fundamental level, heart failure with a preserved EF may be considered as a primary disorder of filling (diastolic dysfunction), and heart failure with a reduced EF, may be considered as a primary disorder of emptying (systolic dysfunction) (Table 1.1). As gender differences in heart failure are often characterised by differences in the prevalence of heart failure with a reduced or preserved EF, in the following section I will briefly address the fundamental differences between heart failure with a reduced versus preserved EF.

1.2.1 Heart failure with a preserved ejection fraction

Heart failure with a preserved EF is a previously neglected, yet nevertheless common cause of heart failure (Vasan *et al* 1999), accounting for a significant proportion of morbidity and mortality (Zile *et al* 2005). Heart failure with a preserved EF, often called ‘diastolic heart failure’ to define the essential underlying functional abnormality, was first defined in 1988 to describe a subset of chronic heart failure patients characterised by concentric remodelling with a normal or even a reduced left ventricular (LV) filling volume, and an abnormal LV diastolic function (such as a slowed or delayed relaxation) due to an increased cardiac stiffness (Kessler *et al* 1988). Subsequently, the terms ‘diastolic dysfunction’ and ‘diastolic heart failure’ were recognised. In this regard. diastolic dysfunction was identified as a sub-clinical syndrome associated with an abnormal mechanical property of the heart where the ability of the ventricular myocardium to return to a relaxed state is impaired. Diastolic heart failure was identified as a clinical syndrome with characteristic symptoms and signs of heart failure associated with a relatively normal systolic function but a reduced diastolic function

(Vasan *et al* 1999, Zile *et al* 2004, Zile *et al* 2005, Aurigemma *and Gaasch* 2004). Abnormal diastolic function is detected via measurements of the

Table 1.1. Structural and functional changes in systolic versus diastolic heart failure (Summarized from the references listed below).

Parameters	**Systolic heart failure**	**Diastolic heart failure**
Important		
Ejection fraction	Decreased	Normal
Gender	Predominantly Male	Predominantly Female
End-diastolic volume	Increased	Normal
Left ventricular shape	Spherical	Unchanged
Unimportant		
Left ventricular Mass	Increased	Increased
Left ventricular cavity size	Increased	Increased or normal
Mass/Cavity	Decreased	Increased
Wall thickness	Decreased	Increased
End-systolic stress	Increased	Normal
End-diastolic stress	Increased	Increased
End-systolic volume	Increased	Decreased or normal

Aurigemma *et al* 2006, Chatterjee and Massie 2007, Van Heerebeek *et al* 2006, Dubourg *et al* 2008, Vasan *et al* 1999, Jessup *et al* 2003, Bursi *et al* 2006, McMurray *et al* 2010, Paulus *et al* 2007, Gaasch and Zile 2004, Sanderson *et al* 2007, Zile *et al* 2004, Aurigemma *et al* 2004, Zile *et al* 2005, Gilbert and Glantz 1989.

diastolic ventricular pressure decline, and the relationships between diastolic pressure and volume, and ventricular wall stress and strain (Gilbert and Glantz 1989). These measures detect some of the characteristic features of diastolic dysfunction (Gilbert and Glantz 1989) and diastolic heart failure (Zile *et al* 2004), such as an increased filling pressure for any given volume due to a reduced cardiac compliance or an increased chamber stiffness and concentric remodelling (Chatterjee and Massie 2007) (Figure 1.1). Figure 1.1 depicts the basic functional and structural changes that occur in diastolic dysfunction, such as an increased LV wall thickness, reduced chamber size, and the impact of these changes on the LV pressure-volume relationship. Although there are many known causes of diastolic dysfunction the exact mechanisms by which they cause the characteristic structural and functional changes are only superficially understood, possibly due to the diversity of the heart failure syndrome phenotype. For instance, diastolic dysfunction has been associated with changes in cardiomyocyte sarcomere anatomy (Chatterjee and Massie 2007), collagen regulation and crosslinking (Norton *et al* 1996, Norton *et al* 1997, Badenhorst *et al* 2003a), and myocardial calcium handling (Sordhal *et al* 1973). A reduced cardiac diastolic function, which translates into an increased ventricular filling pressure at a given filling volume (Figure 1.1), may result in an increased left atrial pressure, which subsequently leads to the clinical signs and symptoms of heart failure, such as pulmonary congestion and peripheral oedema. Although the pathophysiology of diastolic heart failure has generally been considered as separate from heart failure with a reduced EF, diastolic dysfunction may progress to systolic dysfunction (Yu *et al* 2002, Redfield *et al* 2003, Hein *et al* 2003).

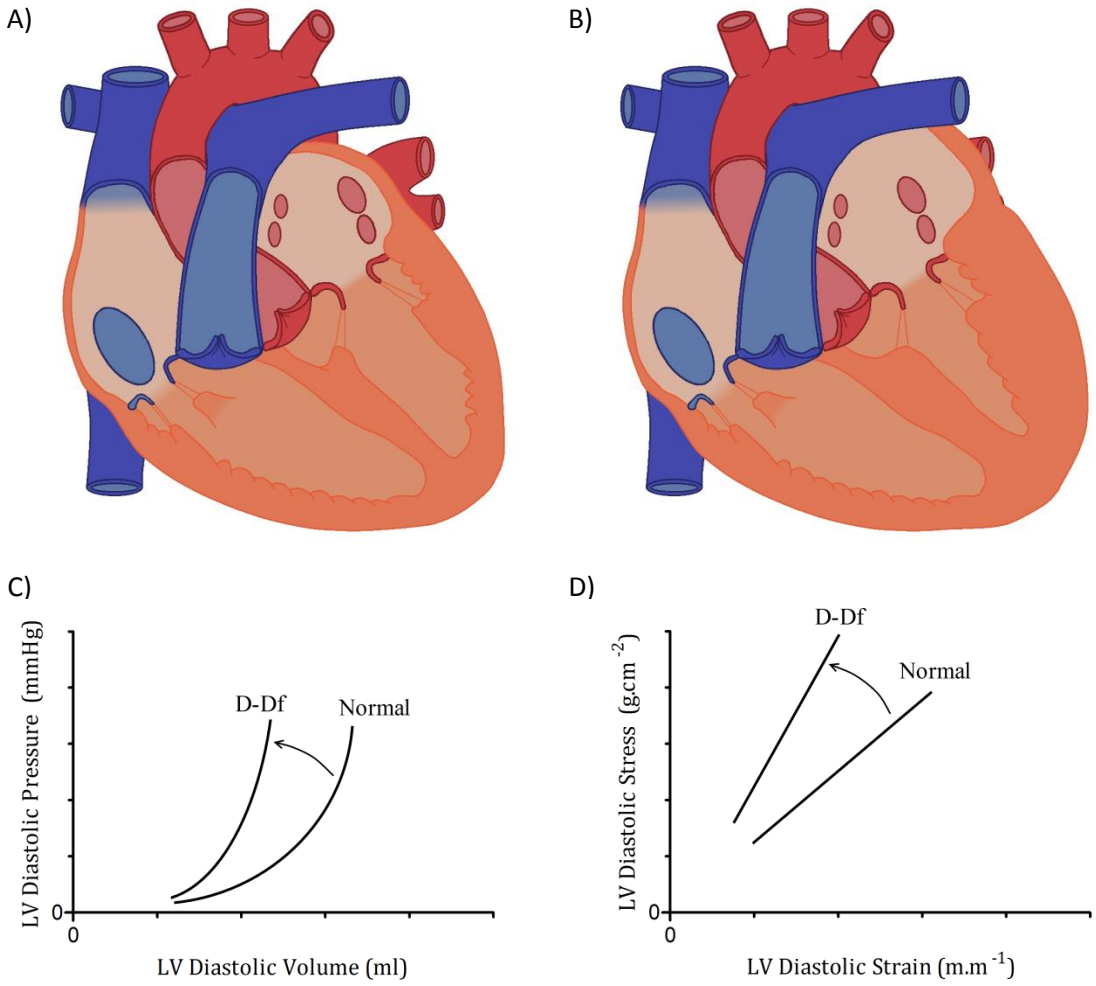


Figure 1.1. Concentric remodelling in heart failure with a preserved ejection fraction. Panel A depicts a structurally normal heart; Panel B depicts a heart with isolated left sided concentric remodelling typical of heart failure with a preserved ejection fraction (depicting no mitral valve pathology); Panel C depicts the shift in the left ventricular (LV) diastolic pressure-volume relationship associated with diastolic dysfunction (D-Df); Panel D depicts the shift in the LV diastolic stress-strain relationship associated with D-Df.

1.2.2. Heart failure with a reduced ejection fraction.

Heart failure with a reduced EF is well recognised form of heart failure (Zile *et al* 2005, Chatterjee and Massie 2007, Aurigemma *et al* 2004, McMurray *et al* 2010). The fundamental functional abnormality of heart failure with a reduced EF is a reduced systolic chamber and myocardial function, which may or may not translate into a decrease in pump function (stroke volume and cardiac output). The reduction in EF may be attributed to decreases in myocardial cellular function, or an increased afterload produced by cardiac dilatation (increased cavity size and a reduced wall thickness which according the Law of LaPlace [Tension is determined by (pressure x radius)/(2 x, wall thickness)] increases wall tension or stress. Indeed, whereas heart failure with a preserved EF is defined by a left shift in the LV diastolic pressure-volume relationship and concentric remodelling (Figure 1.1), heart failure with a reduced EF is defined by a right shift in the LV diastolic pressure-volume relationship and eccentric chamber remodelling (Figure 1.2) (Zile *et al* 2005, Chatterjee and Massie 2007, Aurigemma *et al* 2004, McMurray *et al* 2010). Several possible mechanisms have been put forward to explain the reduction in wall thickness and dilatation in patients with systolic cardiac dysfunction. These include myocyte loss, myocyte dysfunction, inflammatory processes, alterations in the interstitium, and cardiomyocyte sarcomere disruption.

1.2.3 Cardiac dilatation as both cause and consequence of cardiac dysfunction and failure.

As myocardial systolic dysfunction progresses, stroke volume and cardiac output are maintained in-part as a consequence of fluid retention and increases in cardiac volume

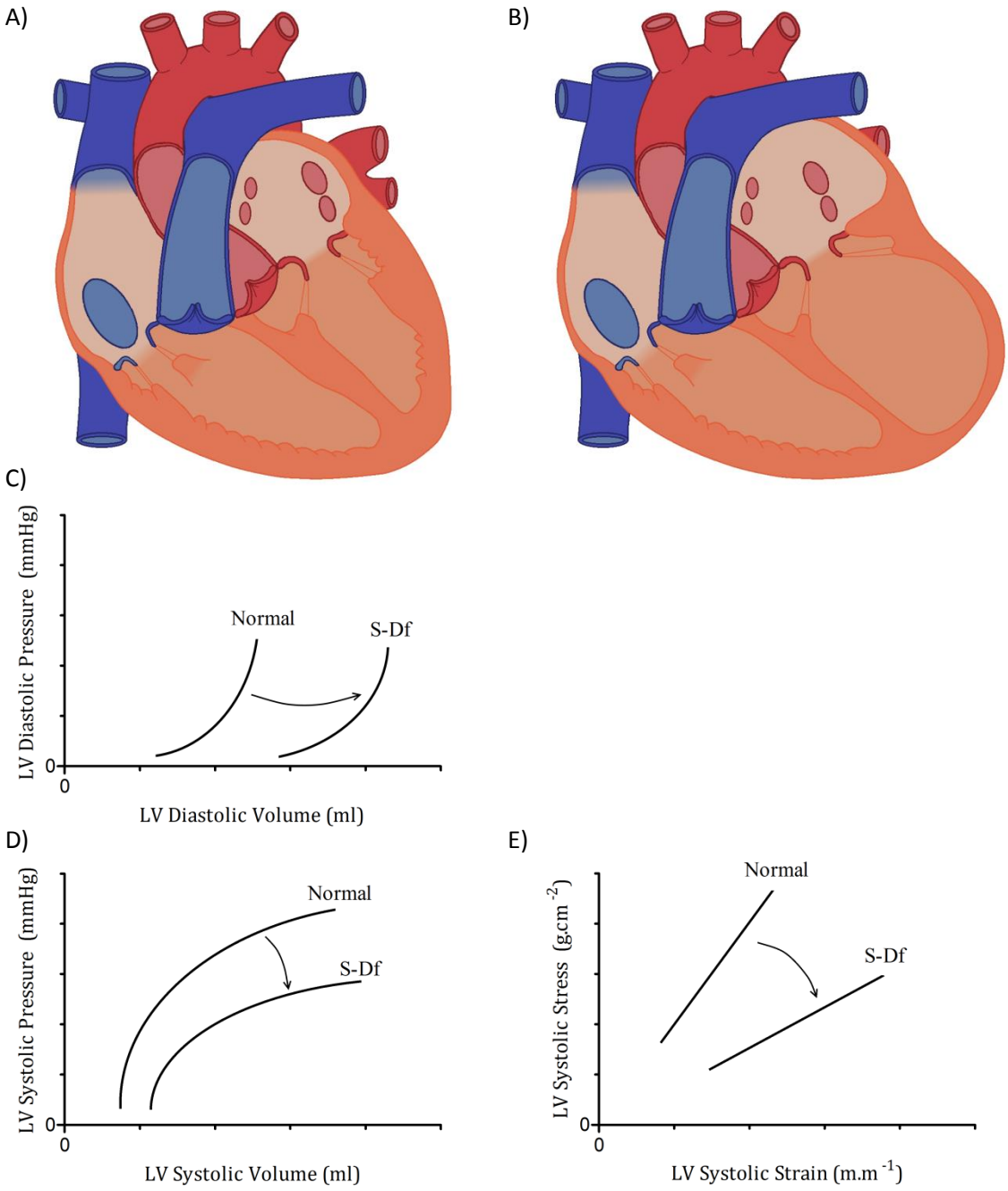


Figure 1.2 Cardiac remodelling in heart failure with a reduced ejection fraction: Panel A depicts a structurally normal heart; Panel B depicts a heart with isolated left sided eccentric (dilatatory) remodelling typical of heart failure with a preserved ejection fraction (with no mitral valve pathology); Panel C depicts the shift in the left ventricular (LV) diastolic pressure-volume relationship during systolic dysfunction (S-Df); Panel D depicts the shift in LV systolic pressure-volume relationship during S-Df; Panel E_{es} depicts the shift in the LV systolic stress-strain relationship during S-Df.

preloads. In this regard, increases in cardiac filling volumes recruit the Frank-Starling effect and hence through an enhanced stretch of the myocardium, increase the force of contraction. However, the deleterious effect of increases in cardiac filling volumes is increases in filling (diastolic) pressures and hence in left atrial pressures. Increases in left atrial pressures contribute toward the development of pulmonary oedema (left heart failure) and the development of right heart failure. What was previously considered as an important compensatory change to maintain low filling pressures in the face of high filling volumes was the development of a right shift in the cardiac diastolic pressure-volume relationship, or cardiac dilatation. However, as indicated in the aforementioned discussion, cardiac dilatation results in an increased wall stress (afterload). A more contemporary notion is therefore that cardiac dilatation contributes toward progressive systolic chamber dysfunction in cardiac disease. What is the evidence to support this notion?

A number of lines of evidence support the view that cardiac dilatation should be viewed as a cause and not just a consequence of cardiac systolic chamber dysfunction. Indeed, in both clinical (Gaudron *et al* 1993, Pfeffer *et al* 1992, Vasan *et al* 1997) and pre-clinical (Veliotes *et al* 2005, Badenhorst *et al* 2003b, Gibbs *et al* 2004, Veliotes *et al* 2010) studies, cardiac dilatation has been noted to precede rather than follow left ventricular systolic chamber dysfunction and clinically relevant heart failure. Additionally, during treatment for heart failure, a reduction in cardiac chamber size and volumes is associated with improved cardiac outcomes, including survival (Doughty *et al* 1997, Sharpe and Doughty 1998, Pfeffer *et al* 1992). Patients with an increased risk for developing cardiac dilatation also have an increased risk for developing heart failure or mortality after 6 months (de Kam *et al* 2002, Nestico *et al* 1985, Gadsboll *et al* 1990, Lee *et al* 1993, Foley *et al* 1995, Foley and Palfrey 1998). Furthermore, in the transition to heart failure in pressure overload states, cardiac chamber dilatation is more closely associated with a reduced systolic chamber

function than decreases in intrinsic myocardial systolic dysfunction (Norton *et al* 2002). The association of cardiac dilatation and cardiac outcomes in heart failure is sufficiently well established that measurements of cardiac chamber dimensions and thus the extent of cardiac dilatation have been added as risk predictors to guidelines for the management of heart failure (Hunt *et al* 2001).

1.2.4. Identification of cardiac dilatation.

Dilatation of the heart increases the internal dimensions of the ventricles, a change which is readily identified in a clinical setting by such techniques as echocardiography or magnetic resonance imaging (MRI). In this regard, cardiac end diastolic volume, which represents the maximum volume during the cardiac cycle, is employed as an index of chamber dilatation. However, as the heart is not an isolated system, and its function is altered by the physiology of the rest of the body (Gilbert and Glantz, 1989), cardiac end diastolic volume is dependent on such factors as preload, afterload, and heart rate. Indeed, an increase in volume preload will increase cardiac end diastolic volume (a greater filling will occur), an increased afterload will decrease systolic function, result in a reduced ventricular ejection and hence also increase end diastolic diameter, and a decreased heart rate will allow for a greater time for filling and hence similarly increase end diastolic volume. While such *in vivo* measures of cardiac end diastolic volumes or diameters are appropriate for a clinical setting, load- and heart rate-independent approaches to identifying cardiac dilatation provide more reliable data. In this regard, the volume intercept of the cardiac diastolic pressure-volume relationship excludes the impact of preload, afterload and heart rate on diastolic diameters. This concept will be further expanded on in the methods chapter of the present dissertation.

1.2.5 Neurohumoral stimulation as a major mechanism responsible for progressive heart failure with a reduced ejection fraction.

Although there is little understanding as to what differentiates whether cardiac disease will progress to heart failure with a preserved or reduced EF, one possible factor that may determine the progression to one or the other form of heart failure is the impact of neurohumoral activation. In this regard, irrespective of the cause of heart failure, neurohumoral stimulation is a well-recognised determinant of progressive systolic cardiac dysfunction and subsequent heart failure (Yoshikawa *et al* 1996, Mann and Bristow 2005). Indeed, a number of findings underpin the neurohumoral hypothesis of progressive heart failure with systolic chamber dysfunction. Patients with heart failure have increased plasma concentrations of noradrenaline, relative to healthy individuals (Thomas and Marks 1978). Plasma concentrations of noradrenaline predict survival in patients with heart failure (Cohn *et al* 1984). Noradrenaline released from the failing myocardium may be approximately 50 times greater than that of non-failing myocardium (Esler *et al* 1997). Increased plasma noradrenaline concentrations are associated with the severity of systolic chamber dysfunction and the degree of cardiac dilatation (Swedburg *et al* 1990, Anad *et al* 203, Francis *et al* 1993, Kluger *et al* 1982). Moreover, adrenergic receptor blockers reduce mortality, increase systolic chamber function and decrease cardiac chamber dimensions in patients with heart failure and a reduced systolic chamber function (Doughty *et al* 2004, Packer *et al* 1996, Packer *et al* 2001, Herlitz *et* al 1999, Lechat *et al* 1997, Lechat *et al* 1999, Domanski *et al* 2003, Poole-Wilson *et al* 2003, Flather *et al* 2005, Butler *et al* 2006, Hernandez *et al* 2009). In contrast, there is evidence to support a lack of beneficial effect of neurohumoral blockade on outcomes in patients with heart failure and a preserved EF (Hamdani *et al* 2009, Borlaug *et al* 2011).

At a preclinical level, there is also significant evidence to demonstrate that neurohumoral activation promotes the development of cardiac dilatation and systolic chamber dysfunction. In this regard, transgenic models of β_2-adrenergic receptor over-expression (Gao *et al* 2003, Freeman *et al* 2001) and chronic adrenergic β-adrenergic receptor stimulation (Woodiwiss *et al* 2001, Badenhorst *et al* 2003, Veliotes *et al* 2005, Osadchii *et al* 2007) produce a cardiomyopathy with systolic chamber dysfunction and marked cardiac chamber dilatation. Hence, there is no question that neurohumoral activation mediates a reduced rather than a preserved systolic chamber function in progressive cardiac disease.

1.3.0 Gender effects on cardiac structure and function.

There are a number of lines of evidence to indicate that gender differences exist in the structure and function of the heart. This evidence exists at a clinical level in patients with heart failure, at a community-based level in otherwise healthy individuals, at a preclinical level in basic cardiac structure and function and in animal models of cardiac dysfunction and heart failure. In the following section I will review this evidence.

1.3.1 Gender differences in human heart failure.

There is considerable evidence to indicate that marked gender differences occur in the pathophysiological features that characterise heart failure (Regitz-Zagrosek and Seeland 2011, Konhilas 2010, Chin and Goldman 1998, Adams et al 1999, Tamura et al 1999, Deschepper and Llamas 2007). In this regard, consistent and striking differences have been reported in the proportion of men and women with heart failure with a reduced EF. A number

of these studies have been summarised in Table 1.2. In this regard, far more men develop heart failure with a reduced

Table 1.2 The proportion of men versus women included in heart failure studies.

		Sample number			% Men
Article	**Study name**	**Total**	**Men**	**Women**	
Heart failure with a reduced left ventricular ejection fraction					**Average - 75%**
					Excluding ALLHAT - 77%
Adams *et al* 1998.	FIRST	471	359	112	76%
Davis *et al* 2008.	ALLHAT	506	315	191	62%
Ghali *et al* 2003	BEST Study	2708	2115	593	78%
Lewis *et al* 2007	Israel Nationwide Heart Failure Survey	1481	1045	436	71%
Rathore *et al* 2002	Digitalis Investigation Group trial	6800	5281	1519	78%
Ghali *et al* 2002	MERIT-HF	3991	3093	898	78%
Simon *et al* 2001.	CIBIS II	2647	2132	515	81%
Heart failure with either a reduced or normal left ventricular ejection fraction.					**Average 52.8%**
Chin and Goldman 1998.	---	179	89	90	50%
Davis *et al* 2008.	ALLHAT	910	511	399	56%
Kalogeropoulos *et al* 2009.	Health ABC study	258	124	134	48%
Levy *et al* 2002.	Framingham Heart Study	1075	527	548	49%
Lewis *et al* 2007.	Israel Nationwide Heart Failure Survey	2845	1603	1242	56%
Senni *et al* 1998.	---	216	125	91	58%

ALLHAT: Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial; BEST: Beta-Blocker Evaluation of Survival Trial; CIBIS II: Cardiac Insufficiency Bisprolol Study; FIRST: Flolan International Randomized Survival Trial; Health ABC: The Health, Aging, and Body Composition Study; MERIT-HF: Metoprolol Extended-release Randomized Intervention Trial.

EF as compared to women (Table 1.2, upper section). Importantly, these gender differences cannot be attributed to gender differences in the development of heart failure *per se* as an equal proportion of men and women develop heart failure (Table 1.2, lower section). As similar numbers of men and women develop heart failure, but more men develop heart failure with a reduced EF, it stands to reason, that more women and less men are prone to develop heart failure with a preserved EF.

Not only are men more prone to developing heart failure with a reduced rather than preserved EF, but men with heart failure with a reduced EF have a worse outcome. Indeed, in the Flolan International Randomized Survival Trial (FIRST), consisting of 359 men and 112 women with marked symptoms of heart failure, and a severe reduction in LV EF, the relative risk for death for male versus female subjects was 3.08 for subjects with a non-ischaemic aetiology (Adams *et al* 1998). Further, in the same study, the relative risk for death of male versus female subjects with an ischaemic aetiology was 1.64. Results from the Cardiac Insufficiency Bisprolol Study (CIBIS II), where 2132 men and 515 women with New York Heart Association (NYHA) class III and IV heart failure were analysed, women had significantly lower mortality rate relative to men (Simon *et al* 2001). Subsequent work has substantiated the notion that men with heart failure and a reduced EF have a greater mortality than women (Vasan *et al* 1999, O'Connor *et al* 2012). In contrast, women with heart failure and a preserved EF may have a greater mortality than men (Lewis *et al* 2007). Hence, there is some evidence that gender determines outcomes in heart failure, such that in heart failure with a reduced EF, men have greater mortality than women, while in heart failure with a preserved EF, women have a greater mortality than in men.

There is also significant evidence to suggest that men and women demonstrate differences in the age of onset of heart failure. In this regard in the Framingham Heart Study, 331 men and 321 women were diagnosed with heart failure at the respective average ages of

68.1 (SD:10.6) and 71.9 (SD:10.6) years ($p<0.001$ for the age difference) (Ho *et al* 1993). Similarly, in a community- based population study, 378 men, and 289 women were diagnosed with heart failure at the ages of 73 (95% CI of 12) and 79 (95% CI of 11) years respectively ($p<0.001$ for the age difference) (Roger *et al* 2004). Furthermore, as part of the OPTIMIZE-HF registry analysis (Fonarow *et al* 2007) heart failure with a reduced EF was diagnosed in 20118 people at an average of 70.4 (SD-14.3) years of age, of which, 68% were men, while heart failure with a preserved EF was diagnosed in 21149 people at an average age of 75.1 (SD-13.1) years, of which only 42% were men. Similarly, as part of the Israeli Nationwide Heart Failure Survey, heart failure with a reduced EF was diagnosed in 1481 people at an average age of 71 (SD-12) years, of which 61% were men, while heart failure with a preserved EF was diagnosed in 1364 people at an average age of 73 (SD-12) years, of which, 52% were men (Lewis *et al* 2007). Hence, not only is there a gender difference in the form of heart failure that develops, but also a difference in the age of onset, such that men develop heart failure with a reduced EF at a younger age, while women develop heart failure with a preserved EF and are typically older. Could these gender differences in heart failure be attributed in-part to differences in the management or response to therapeutic agents in men and women with heart failure?

1.3.2 Are there gender differences in the response to treatment in heart failure?

It is now well-acknowledged that women are generally under-represented in clinical studies of β-blocker therapy and that few such studies appropriately stratify the analysis by sex to allow for the detection of a gender difference (Frankenstein *et al* 2012). Further, there is little evidence comparing gender effects on the impact of β-adrenergic receptor blockers in heart failure with a preserved versus reduced EF. In this regard, when stratified analyses are

available, data are either stratified only by sex (Ghali *et al* 2002, Ghali *et al* 2003, Simon *et al* 2001), or by EF (Fonarow *et al* 2007, Van Veldhuisen *et al* 2009), but not by both. Nevertheless in an analysis of the CIBIS-II study in which only patients with a reduced LV EF were enrolled, women treated with the adrenergic receptor blocker bisoprolol had a lower (6% vs 12%, p=0.01) mortality relative to men, and this gender difference did not exist in the placebo group (13% vs 18%, p=0.10) (Simon *et al* 2001). Importantly, the relative risk of mortality for women compared to men with an underlying ischaemic aetiology was 0.63 (95%CI 0.39-1.02, p=0.057), but there was no such sex relationship in non-ischaemic patients (p=0.734) (Simon *et al* 2001). In the BEST study (Ghali *et al* 2003), where patients with heart failure with a reduced EF were treated with the adrenergic receptor blocker bucindolol, women also had a reduced mortality relative to men (27% and 33% mortality respectively, p=0.02). Yet, in this study it was noted that the gender effect was confined to a non-ischaemic aetiology, lower NYHA class (III vs IV), or non-diabetic patients (Ghali *et al* 2003). Importantly, 64% of the men enrolled had an ischaemic aetiology, while 62% of the women enrolled had a non-ischaemic aetiology (p<0.001 for the difference) (Ghali *et al* 2003). Further, Ghali *et al* (2002) performed a similar analysis on the MERIT-HF study population, in which 898 women and 3093 men with heart failure participated, and where the adrenergic receptor antagonist metoprolol was noted to decrease the relative risk of death in both men (18% relative to placebo, p=0.011) and women (21% relative to placebo, p=0.044). However, a direct comparison of the difference in survival between men and women was only performed in the placebo group, but did reveal that women had a significantly lower risk of death relative to men (0.63, 95%CI 0.43-0.91, p=0.015) (Ghali *et al* 2002).

Although there is some evidence to suggest that gender differences may exist in the response to β-adrenergic blocker therapy in patients with a reduced EF, there is almost no evidence to show whether such sex differences characterise effects in heart failure with a

preserved EF. In this regard, in a small (n=66 patients) recent study (Farasat *et al* 2010), the effects of β-blocker therapy on rehospitalisation in patients with a preserved EF was evaluated, and the authors reported an increased risk of rehospitalisation in women but not men.

1.3.3 Gender differences in basic cardiac structure.

There are a number of lines of evidence to suggest that irrespective of gender differences in cardiac pathology and treatment, differences may exist between men and women in the basic structure of the heart. As such, differences in structure and function may in-part account for gender differences in cardiac function when pathology occurs or to the response to treatment. To that effect, as part of the Dallas Heart Study in 1183 men (44±9 years of age) and 1435 women (45±9 years of age) whom were otherwise healthy, men had a greater LV mass (LVM) than women (191.7±44.4 vs 141.3±33.8 respectively; mean±SD; $p<0.001$), regardless of adjustment for body surface area ($p<0.001$) (Chung *et al* 2006). In addition, in 400 men and 400 women from the Multi-Ethnic Study of Atherosclerosis (MESA) an increased LVM and LV volume were noted in men relative to women ($p<0.0001$) regardless of indexing for height, body mass index (BMI), body weight, or body surface area (Natori *et al* 2006). In a community cohort over the age of 45 years, LV end diastolic volume was higher in men than in women regardless of adjustments for body surface area ($p<0.0001$), and LV end diastolic volume was positively correlated with age in men ($r=0.13$; $p=0.003$) but not in women ($r=0.0$; $p=0.99$) (Redfield *et al* 2005). Importantly, in that study (Redfield *et al* 2005) both LVM and LV relative wall thickness (an index of the degree of LV concentric remodelling) increased to a greater degree with age in women as compared to men ($p<0.0001$ for both). Thus, men appear to have a greater left ventricular volume, diameter,

and mass relative to women, but women appear to have a greater degree of LV concentric remodelling. Hence, men may be at risk of developing eccentric LV remodelling following a myocardial pathological insult, a change which may ultimately translate into heart failure with a reduced EF, whilst women may be at risk of developing concentric LV remodelling following a myocardial pathological insult, a change which may result in heart failure with a preserved EF.

1.3.4 Gender differences in cardiac structure in animal models

In human studies, gender differences in cardiac structure are confounded by an inability to follow individuals over the course of a lifetime, control for the severity and timing of pathological events, account for treatment differences or the impact of genetic or environmental factors on the heart, or control for the time between initiation of a pathological event and admission for therapy. In contrast, these factors can be readily controlled in animal studies. What is the evidence from animal studies that gender influences basic cardiac structure?

Healthy male rats have been demonstrated to have a higher LVM (1.04±0.22 g; mean±SD) than female rats (0.67±0.13 g; p=0.01 for the difference), but with adjustment for body mass (2.6±0.6 $g.g^{-1}$ vs 2.3±0.4 $g.g^{-1}$; respectively), or for tibial length (0.22±0.07 $g.mm^{-1}$ vs 0.19±0.06 $g.mm^{-1}$; respectively) these differences were eliminated (Forman *et al* 1997). However, LV end diastolic diameters are higher in male (7.7±0.7 mm; mean±SD) than in female (6.4±0.6 mm; $p<0.001$) rats, regardless of adjustment for body mass ($p<0.003$) and male rats have a reduced LV relative wall thickness, also regardless of adjustment for body mass (Forman *et al* 1997). Thus, in healthy rodents at least, gender differences in eccentric LV remodelling cannot be accounted for by differences in body size or LVM. In a study

conducted to evaluate the impact of aortic banding on the LV, LV diastolic diameters were also noted to be larger in the male sham-operated group (mean±SEM) (8.2±0.2 mm) as compared to the female sham-operated group (7.0±0.1 mm) (Douglas *et al* 1998). Hence, there is some evidence from animal studies to suggest that basic LV chamber sizes is larger in males as compared to females and that this is not entirely accounted for by differences in body size.

1.3.5 Gender differences in basic cardiac function in human studies

As described in sections 1.3.3 and 1.3.4, as compared to females, males have greater LV internal diameters, changes which are not necessarily determined by a greater body size. Hence, as compared to women, men are predisposed to eccentric LV changes. Do these geometric LV changes translate into functional differences? In this regard, a number of studies have provided the evidence to show that cardiac systolic chamber function is reduced in men as compared to women.

In earlier studies no gender differences in left ventricular endocardial fractional shortening were noted in 18 to 54 year old subjects but after the age of 55 years men had a lower LV endocardial fractional shortening ($p<0.05$) than women, even when adjusted for end systolic wall-stress ($p<0.01$) (Simone *et al* 1991). These data (Simone *et al* 1991) suggested that LV systolic function was lower in elderly men than in woman, but that the eccentric LV remodelling process, which results in a higher wall stress, could not explain these changes. However, subsequent studies suggested that the LV eccentric remodelling process may play a role. Indeed, in 102 men, and 141 women, LV EF (mean±SD) was noted to be lower in men (62±7%) as compared to women (64±6%; $p=0.03$ for the difference) (Celentano *et al* 2003). This occurred despite a higher LV mid-wall fractional shortening (an

index of myocardial function) in men (17.4±2.2) as compared to women (16.1±2.2; p=0.02 for the difference) (Celentano *et al* 2003). As myocardial function appeared intact, these data (Celentano *et al* 2003) suggested that gender differences in LV EF are accounted for by the eccentric LV remodelling process. However, further studies did not support these findings. Indeed, in a larger study sample of 490 men, and 861 women in the Strong Heart Study, although men were reported to have a reduced LV EF (mean±SD) (63±9%) relative to women (66±8%; p=0.002 for the difference), and a decreased LV endocardial fraction shortening (34±7% for men, vs 36±6% for women; p=0.002), men also had a lower rather than higher LV mid-wall fractional shortening (17±3%) than women (18±2%; p=0.003 for the difference) (Bella *et al* 2006). Nevertheless, unlike the study showing a higher LV mid-wall fractional shortening together with a reduced EF in men as compared to women, where LV volumes were also increased (Celentano *et al* 2003), in the Strong Heart Study population LV volumes were similar in men as compared to women (Bella *et al* 2006). Moreover, in a large population based-study conducted in 1069 men, and 1115 women, where men had a reduced LV EF as compared to women (64.4±8.2% for men, vs 66.88±7.66% for women; p<0.001) in those less than 50 years of age the decreases in EF in males were not associated with parallel differences in LV systolic stress (Claessens *et al* 2007).

However, all of the aforementioned studies were performed using echocardiography, which results in a reduced accuracy and precision of LV structural and functional changes as compared to MRI. What is the evidence for gender differences in function obtained from MRI-based studies? In this regard, MRI studies conducted in 400 men, and 400 women, also demonstrated that men have a lower LV EF than women (p<0.001), regardless of adjustment for body weight (p<0.001), body surface area (p<0.001), height (p<0.001), or BMI (p<0.001) (Natori *et al* 2006). In this regard, men also had higher LV volumes than women even with adjustments for differences in body size (Natori *et al* 2005). However, Natori *et al* (2006)

failed to report on gender differences in LV wall stress. Similarly, using MRI based measurements of LV structure and function, in the Dallas Heart Study, Chung *et al* (2006) demonstrated a decreased LV EF in men (70%) relative to women (75%; $p<0.01$). Subsequently, in an analysis of MRI-based measurements in the MESA population of 5004 subjects without signs of cardiovascular disease, associated with increases in LV volumes, men were noted to have a lower LV EF as compared to women across all age groups from 40 to 75 years of age (Cheng *et al* 2009).

Therefore, in summary, current evidence consistently shows that as compared to women, men have a reduced LV EF, a finding that cannot be explained on the basis of differences in body size, but may to some degree be related to eccentric LV remodelling. These data suggest that the greater prevalence of heart failure with a reduced LV EF in men as compared to women (Table 1.2, upper panel) may be in-part a result of the pre-existing lower LV EF and eccentric LV remodelling in men before the development of heart failure.

1.4. Gender differences in preclinical studies of cardiac disease.

As the gender-differences that exist in human heart failure or in the human heart prior to the development of heart failure may be confounded by multiple variables including differences in the timing of the presentation of heart failure or its risk factors, the severity of the underlying pathology (e.g., severity of coronary artery disease), differences in the cause of the heart failure or cardiac function (e.g. coronary artery, hypertensive, diabetic, obesity-related heart disease), variations in management strategies, etc, the question arises as to whether similar sex-differences exist in animal models of heart failure or cardiac disease where these factors are controlled for. What is this evidence?

1.4.1 Gender differences in preclinical studies of cardiac pathology attributed to pressure and volume overload.

Cardiac pressure (such as occurs with hypertension or aortic stenosis), and volume (such as occurs with regurgitant valves or high output states) overload are well-recognised as discrete causes of cardiac systolic chamber dysfunction and heart failure. Pressure overload states increase the afterload that the heart must work against, resulting in a greater degree of LV concentricity, whilst volume overload increases the preload on the heart, resulting in a greater degree of LV eccentricity (Toischer *et al* 2010). Do animal studies provide evidence to support gender differences in the LV response to pressure or volume overload?

With respect to pressure overload states, Douglas *et al* (1998) subjected both male and female weanling Wistar rats to supravalvular aortic banding for 20 weeks. Male rats clearly developed a greater degree of eccentric LV remodelling than females rats, such that male rats LV anterior and posterior wall thickness adjusted for body mass was lower than that of female animals and LV end diastolic diameters were higher in males as compared to females (Douglas *et al* 1998). However, male rats failed to develop a greater reduction in LV endocardial fractional shortening (Douglas *et al* 1998). In contrast, in a more recent study, male mice subjected to pressure overload developed a lower LV EF than female mice (Montalovo *et al* 2012). Moreover these gender-specific effects of pressure overload on LV EF were associated with parallel but inverse gender-specific changes in LV volumes (Montalovo *et al* 2012).

With respect to volume overload states, Gardner *et al* (2002) compared the actions of an arterio-venous shunt on male and female Sprague Dawley rats. In this regard, a higher mortality rate was noted in male (24.5% mortality of the 200 male rats in the study) than in the female (2.5% mortality of the 40 female rats in the study) rats, and males, but not females

had evidence of LV eccentric remodelling (greater increase in LV end diastolic diameter), LV systolic chamber dysfunction and pulmonary congestion (lung weights) (Gardner *et al* 2002). Subsequently, Dent *et al* (2010, 2012) similarly demonstrated that as compared to female rats, male rats exposed to an arterio-venous fistula develop a reduced LV endocardial fractional shortening and an increased LV end diastolic pressure.

In summary, consistent with data obtained in human studies, irrespective of whether pressure or volume overload states are studied, male as compared to female rodents, are susceptible to reductions in LV systolic chamber function and pathological evidence of heart failure in association with LV eccentric remodelling, which may or may potentially precede the functional changes (Douglas *et al* 1998).

1.4.2 Gender differences in preclinical studies of myocardial infarction-induced cardiac remodelling.

Myocardial infarction (MI) is a common cardiovascular event, often resulting in adverse cardiac remodelling with subsequent heart failure. There are clear gender differences in the degree of mortality, with male animals having a greater mortality than that of females (Cavasin *et al* 2006). However, are these gender differences in mortality in-part attributed to gender differences in adverse cardiac remodelling that occurs post-MI?

In this regard, following ligation of the left coronary artery, male rats developed a greater increase in LV diameter, an increased LV free wall area, and a greater right shift in the LV end diastolic pressure-volume relationship relative to female rats (Jain *et al* 2002). However, no comparisons of LV systolic function between genders were performed in that study (Jain *et al* 2002). Following ligation of the left coronary artery, Wu *et al* (2003) also demonstrated a greater increase in LV diastolic diameter in males as compared to females.

Although LV EF was similar between male (40±1%) and female (41±1%) animals at two days post ligation, 28 days post ligation, males had a decreased LV EF (30±1%; $p<0.05$) while females had no significant decreases in LV EF (37±2%) (Wu *et al* 2003). Hence, gender-specific effects of MI on LV systolic chamber function cannot be attributed to differences in tissue damage produced by the MI, or to acute LV remodelling, but rather to the chronic remodelling process. Subsequently, Cavasin *et al* (2003, 2004) also demonstrated that after ligation of the left coronary artery, male rats have a higher rate of mortality as compared to female rats, and that male rats develop higher LV internal diameters, and a lower LV EF as compared to female rats. Thus, current evidence suggests that male rats are more susceptible than female rats to post-MI adverse LV remodelling, LV systolic chamber dysfunction and hence to a worse mortality.

1.4.3 Gender differences in preclinical studies of neuro-humoral induced adverse cardiac remodelling and systolic dysfunction.

As indicated in a previous section of the present dissertation (section 1.2.5), irrespective of the cause of heart failure, neurohumoral stimulation is a well-recognised determinant of progressive adverse cardiac remodelling, systolic chamber dysfunction, and subsequent worsening heart failure and poor outcomes in humans (Yoshikawa *et al* 1996, Mann and Bristow 2005). However, evidence for a gender-specific effect on the adverse effects of neurohumoral stimulation on the human heart are lacking, largely because it is impossible to control for confounding factors. The question therefore arises as to whether there is evidence for gender differences in adverse cardiac remodelling and systolic chamber function from animal-based studies?

Following excessive β_2-adrenergic receptor expression in mice, although at 6 months of age no differences in the degree of LV dilatation were noted between male and female animals, at 9, 12 and 15 months of age while both genders had increases in LV diameters ($p<0.01$), only males had a decrease in LV wall thickness ($p<0.05$) and a concomitant increase in LV diameter divided by wall thickness) (an index of eccentric LV remodelling) (Gao *et al* 2003). Further, while LV endocardial fractional shortening decreased in both genders due to β_2-adrenergic receptor overexpression, males developed a lower LV endocardial fractional shortening as compared to females at 9, 12, and 15 months of age (Gao *et al* 2003). While at 15 months of age, transgenic male animals suffered an 88% mortality, female animals suffered only a 44% mortality ($p<0.001$) (Gao *et al* 2003). In a further study of murine β_2-adrenergic receptor overexpression, at 16 months of age, male transgenic animals had a higher (90%) mortality than females (50%, $p<0.01$), and transgenic males developed LV dilatation (LV end diastolic diameter indexed for body mass) earlier than females ($p<0.001$) (Thireau *et al* 2010). Furthermore, male transgenic mice had a higher prevalence of pleural effusions (71% in males vs 37% in females; $p<0.05$) and lung congestion (68% in males vs 37% in females; $p<0.05$), pathological signs of left heart failure, than females (Thireau *et al* 2010). Thus, a possible mechanism that may explain gender differences in adverse LV remodelling and systolic LV chamber dysfunction previously alluded to in aforementioned discussion, is a susceptibility of males to the adverse effects of adrenergic activation on the heart.

1.5.0 Explanation for gender-differences in cardiac structure and function.

An obvious question which arises from studies demonstrating gender differences in heart failure, cardiac pathology prior to the development of heart failure, and basic cardiac

structure and function, is whether these differences can be explained by variations in sex hormones and their actions on the heart. What is the evidence to suggest that sex hormones may explain gender differences in heart failure, cardiac pathology prior to the development of heart failure, and basic cardiac structure and function?

1.5.1 Do differences in sex steroids explain gender differences in cardiac structure and function?

The implications of the evidence for gender differences in cardiac structure and function reviewed in prior sections of the present dissertation is that either testosterone may have deleterious effects or that oestrogen may have beneficial effects on the LV eccentric remodelling process and hence on LV systolic chamber function. What is the evidence for these hypotheses?

With respect to the possible deleterious role of testosterone on eccentric LV remodelling and LV systolic function, contradictory evidence exists. In favour of a deleterious effect of testosterone is the evidence that castration reduces the extent of increase in LV cavity dimensions associated with cardiac β_2-adrenergic receptor over-expression (Gao *et al*. 2003) and aortic constriction (Montalvo *et al*. 2012) in mice. Furthermore, in male rats subjected to coronary artery ligation, castration maintained a greater LV EF, and reduced the degree of LV dilatation (Cavasin *et al* 2003). Further, when female rats subjected to coronary artery ligation received testosterone treatment, LV diameters increased and LV EF was reduced (Cavasin *et al* 2003). However, against a deleterious role of testosterone in mediating eccentric LV remodelling and LV systolic function, is the evidence that castration/orchiectomy worsens increases in LV diameter in rat models of doxorubicin-induced LV dysfunction (Sun *et al*. 2011) and cardiomyocyte necrosis (Kang *et al*. 2012), or

produces no effect on LV internal diameter post-myocardial infarction (Nahrendorf *et al.* 2003). With respect to the possible beneficial role of oestrogen on eccentric LV remodelling and LV systolic function, there is little evidence to support such a role. In this regard, oophorectomy failed to modify mortality, LV diameters or LV systolic chamber function in female mice with β_2-adrenoreceptor over-expression (Gao *et al* 2003). Although there are many studies which investigate the progression and pathology of various models of heart failure and pump dysfunction, few include both male and female animals. Further, those that include both male and female animals do not appropriately stratify the analysis by gender. Hence, in summary there is no evidence to support a beneficial role of oestrogen as a potential mediator of gender differences in the LV eccentric remodelling process and hence in LV systolic chamber function. Moreover, the evidence in favour of testosterone mediating the gender differences in the LV eccentric remodelling process and hence in LV systolic chamber function is contradictory, with some studies suggesting a deleterious action, whilst others do not support this adverse effect.

1.5.2 Interactions between adrenergic stimulation and gender hormones may in-part explain testosterone effects on cardiac structure and function.

One possible mechanism that may explain gender differences in the LV eccentric remodelling process and hence in LV systolic chamber function, is through possible interactions with the adverse effects of sympathetic activation. Indeed, as reviewed in section 1.2.5 sympathetic activation is a major determinant of LV dilatation and hence LV systolic chamber dysfunction. Evidence to suggest an interaction between adrenergic stimulation and gender or sex hormones is summarised in Table 1.3. In this regard, whether testosterone has beneficial or deleterious actions depends largely on whether baseline adrenergic function or

Table 1.3 Effects of testosterone on adrenergic stimulation of the heart.

Article	**Model**	**Main Outcome**
Gao *et al* 2003	Chronic - Mice	Reduced mortality and morbidity in males, due to gonadectomy and thus testosterone removal.
Sun *et al* 2011	Chronic - Male Rats	Testosterone manipulation alters β-2 and β -3 adrenergic receptor expression.
Engelhardt *et al* 1999	Acute – mice	Reduced mortality and morbidity in males, due to gonadectomy and thus testosterone removal.
Wang *et al* 2012	Acute - Male Rats	Increasing testosterone concentrations reduced the noradrenaline release from the myocardium.
Tsang *et al* 2008	Acute - Male Rats	The presence of testosterone reduced the detrimental impact of ischaemic reperfusion combined with adrenergic stimulation.
Vizgirda *et al* 2002	Acute – Rats	Healthy male myocytes had a greater shortening response, and adrenergic receptor density than female myocytes.
Tsang *et al* 2009	Chronic - Male Rats	Testosterone replacement prevents changes in adrenergic responsiveness.
Coulson and Cockroft 2011	Acute – Human	The ß-blocking agent esmolol attenuated the rise in mean arterial pressure in men but not in women.
Kang *et al* 2012	Acute - Male Rats	Testosterone removal was associated with worse adrenergic induced cardiac outcomes.

pathological conditions are being considered. With respect to baseline function, relative to female rat cardiomyocytes, male rat cardiomyocytes have a greater adrenergic receptor density, and a greater contractile responsiveness to adrenergic stimulation (Vizgirda *et al* 2002). Indeed, gonadectomy results in reduced myocardial adrenergic responsiveness, which is ameliorated by supplementation with physiological doses of testosterone (Tsang *et al* 2009). Thus, the presence of testosterone is essential for maintenance of a normal adrenergic responsiveness in the heart. However, studies investigating the relationship between gender and adrenergic stimulation in pathology, report potentially beneficial or detrimental actions of male gender and testosterone depending on the pathology involved. What is this evidence?

Testosterone reduces noradrenaline release from the myocardium induced by ischaemic-reperfusion injury (Wang *et al* 2012). These anti-adrenergic actions of testosterone may be beneficial as gonadectomised rats receiving physiological doses of testosterone develop a reduced myocardial infarct size in a mixed model of ischaemic-reperfusion and excessive adrenergic stimulation (Tsang *et al* 2008). After four weeks of volume overload induced via an arterio-venous fistula, in which eccentric LV remodelling was clearly detected in male but not female rats, a reduction of β_1-adrenergic receptor mRNA expression was noted in male rats as compared to their controls, whilst female rats had no such reduction (Dent *et al* 2010, Dent *et al* 2011). Furthermore, β_2-adrenergic receptor mRNA expression was increased in female hearts relative to control animals, but not in males and β_1 and β_2-adrenergic receptor density and concentration was reduced in male hearts, whilst they were conversely increased in female hearts (Dent *et al* 2011).

With respect to the role of testosterone in contributing toward the deleterious actions of chronic excessive adrenergic activation, as mentioned above, in two separate studies of transgenic mice with β_2-adrenergic receptor overexpression, male mice have been noted to have a greater mortality, increased LV diameters and a reduced LV EF relative to the female

mice (Gao *et al* 2003, Thireau *et al* 2010). Furthermore, gonadectomy in male mice reduces mortality and LV diameters and increases LV EF (Gao *et al* 2003, Thireau *et al* 2010). Hence, at least in the context of excessive β_2-adrenergic receptor over-activation, testosterone interacts with the adrenergic stimulus to enhance the adverse effects of adrenergic activation on adverse LV remodelling and LV EF.

Lastly, as outlined in section 1.3.2, blockade of excessive adrenergic stimulation in men and women with heart failure and a reduced LV EF, appears to have different effects in men and women with women responding better than men. Further, β-blocker therapy might have differing actions on vascular function in men and women (Coulson *et al* 2011). In this regard, esmolol, a cardio-selective β_1-adrenergic blocker, elicits a greater increase in systolic, diastolic, and mean arterial blood pressure in woman that it does in men (Coulson *et al* 2011).

1.5.3 Testosterone deficiency and subsequent testosterone replacement therapy in human heart failure.

Further evidence that castes light on the conundrum of whether testosterone has beneficial or deleterious effects in cardiac disease comes from recent evidence of relationships between heart failure and circulating testosterone concentrations and between circulating testosterone concentrations and outcomes. In this regard, an androgen deficiency in men with heart failure is well documented (Jankowska *et al* 2006, Guder *et al* 2010, Kontoleon *et al* 2003, Pugh *et al* 2003, Wu *et al* 2011). Furthermore, a lower plasma androgen concentration is an independent predictor of poor prognosis in men with heart failure (Volterrani *et al* 2012, Wu *et al* 2011). Indeed, an increased mortality was noted in the lower tertiles of both free ($p=0.008$) and total ($p=0.003$) plasma testosterone concentrations in 175 men diagnosed with congestive heart failure (Wu *et al* 2011). Moreover, plasma free

testosterone concentrations are reduced in men with an LV EF less than 35% (5.8±2.7 $pg.ml^{-1}$; n = 50) as compared to those with an LV EF greater than 35% (6.9±3.3 $pg.ml^{-1}$; n = 465; $p<0.05$) (Davoodi *et al* 2010). Further, Wu *et al* (2011) noted that both free and total testosterone plasma concentrations correlate with LV EF ($r=0.31$; $p<0.01$, and $r=0.30$; $p<0.01$ respectively). In summary, in contrast to aforementioned data obtained in animal studies suggesting a deleterious action of testosterone on LV eccentric remodelling and LV EF, and data demonstrating a worse LV EF values in men as compared to women, findings which suggests that testosterone may have deleterious effects on the heart; data on testosterone concentrations in heart failure suggest that to maintain LV EF, ideal testosterone concentrations may be required.

As a consequence of the findings demonstrating that testosterone deficiency in heart failure has adverse effects, several studies have investigated the effects of testosterone replacement therapy on LV structure and function in men with or without heart failure (Malkin *et al* 2006, Hai-Yun *et al* 2011, Caminiti *et al* 2009). These studies have nevertheless produced contradictory results. In this regard, testosterone replacement therapy given for 12 months to males with heart failure increased LV internal length (Malkin *et al* 2006) and testosterone administration for 1 month to healthy males increased LV end systolic diameter (Chung *et al* 2007). However, 12 weeks of long acting testosterone supplementation did not alter LV end diastolic diameter or improve LV EF in men with congestive heart failure (Caminti *et al* 2009).

Although unlikely to produce direct benefits to the heart, testosterone replacement therapy may nevertheless produce indirect benefits to cardiac function in heart failure. Indeed, testosterone supplementation may improve vascular resistance in either healthy men or men with heart failure (Pugh *et al* 2003, Malkin *et al* 2006) a finding that could translate into long-term benefits in heart failure through reductions in LV afterload. The ability of

testosterone to produce coronary vasodilation has also been well described (Jones *et al* 2004, Malkin *et al* 2010) and this may similarly translate into benefits in heart failure by improving myocardial blood supply.

Testosterone replacement therapy in men with heart failure may also have a number of non-cardiac benefits. Indeed testosterone replacement therapy may improve exercise capacity and functional strength of the body as a whole by altering lean muscle mass, strength, and endurance (Malkin *et al* 2010, Caminti *et al* 2009). In this regard, 12 weeks of long acting testosterone supplementation increased peak oxygen consumption (13.4±4.4 $ml.kg^{-1}$ per min, to 16.3±1.7 $ml.kg^{-1}$ per min; $p<0.05$), peak exercise workload (78.3±16.0 watts, to 88.2±18.7 watts; $p<0.05$), and maximum voluntary contraction of leg muscles (116.7±26.3 Nm, to 135.6±21.2 Nm; $p<0.05$), while placebo administration produced no such changes (Caminti *et al* 2009). Furthermore, 12 months of testosterone supplementation in men with heart failure resulted in increased shuttle walking distances ($p<0.006$) indicating an increased exercise capacity, as well as an increased dominant handgrip strength ($p=0.04$) (Malkin *et al* 2006).

Hence, although there is no apparent benefit to the heart, there are a number of possible reasons to treat hypogonadal male, heart failure patients with testosterone. Nevertheless, there are concerns about the risks of such treatment. Indeed, testosterone may increase the risk for cardiovascular disease in men, by contributing toward the metabolic syndrome, type II diabetes mellitus, abnormal lipid profiles, and atherosclerosis (Jones *et al* 2010, Traish *et al* 2009a, Traish *et al* 2009b, Traish *et al* 2009c, Rhoden and Morgentaler 2004). Moreover, testosterone therapy may result in polycythaemia, and either benign or malignant prostatic hyperplasia (Rhoden and Morgentaler 2004). Furthermore, as has been discussed in aforementioned sections and will be summarised in the subsequent section, there

is still no resolution as to whether testosterone contributes toward LV dilatation and reductions in LV EF.

1.5.4 Is testosterone therapy safe for use in heart failure? Problem statement

As indicated in preceding discussion, it is well recognised that the severity of increases in LV cavity volumes (LV dilatation) predict outcomes in heart failure and that males more frequently develop heart failure with LV dilatation (Bell *et al* 2013). It is therefore possible that longer periods of testosterone administration to males with heart failure than that currently evaluated (Malkin *et al* 2006, Camaniti *et al* 2009) may have adverse effects that have not presently been recognised. Ambiguity as to whether decreases in testosterone in heart failure have adverse, beneficial or neutral effects on chamber dilatation comes from a number of lines of evidence described in previous discussion. In summary, castration reduces the extent of LV cavity dimensions associated with cardiac β_2-adrenergic receptor over-expression (Gao *et al* 2003) and aortic constriction (Montalvo *et al* 2012) in mice. In contrast, castration/orchiectomy worsens LV diameter in rat models of doxorubicin-induced LV dysfunction (Sun *et al* 2011) and cardiomyocyte necrosis (Kang *et al* 2012), or produces no effect on LV internal diameter post myocardial infarction (Nahrendorf *et al* 2003). Furthermore, testosterone replacement therapy given for 12 months to males with heart failure increases LV internal length (Malkin *et al* 2006) and testosterone administration for 1 month to healthy males increases LV end systolic diameter (Chung *et al* 2007). However, in all of these studies (Gao *et al* 2003, Sun *et al* 2011, Kang *et al* 2012, Montalvo *et al* 2012, Malkin *et al* 2006, Chung *et al* 2007, Nahrendorf *et al* 2003) LV chamber diameters were assessed using contractility, load and heart rate-dependent measures. Hence these findings (Gao *et al* 2003, Sun *et al* 2011, Kang *et al* 2012, Montalvo *et al* 2012, Malkin

et al 2006, Chung *et al* 2007, Nahrendorf *et al* 2003) may have been confounded by the influence of testosterone on the vasculature (hence affecting afterload) or myocardial contractility (Malkin *et al* 2010), or by the variable heart rates reported on in different studies.

1.6 Aim of the present dissertation.

To clarify whether testosterone deficiency influences the extent of LV chamber dilatation in cardiac disease, in the present study I therefore aimed to assess the impact of castration on LV diastolic pressure-volume relations in a rat model of marked LV dilatation induced by chronic adrenergic activation (Woodiwiss *et al* 2001, Osadchii *et al* 2007, Booysen *et al* 2012), where LV dilatation may precede a decreased LV contractility (Osadchii *et al* 2007). In support of the present dissertation, the study has been published (Hodson *et al* 2014).

Chapter 2

Methods

2.1 Study groups

The present study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (clearance number: 2010/04/25). In the present study, 36, male Sprague Dawley rats, weighing 200-250g were assigned to one of four groups. Two groups of 10 rats each were employed to assess the impact of 6 months of chronic adrenergic activation on cardiac structure and function. Chronic adrenergic activation was produced by daily subcutaneous injections of the β-adrenergic receptor (β-AR) agonist, isoproterenol administered at a dose of 0.015mg.kg^{-1} in 0.1 mls.100g^{-1} body weight volume of 0.9% saline vehicle for 6 months. The remaining two groups of 8 rats each received daily injections of the same volume of the saline vehicle of isoproterenol for 6 months. Before beginning daily injections of isoproterenol or the vehicle, one group of isoproterenol-treated rats and one group of saline vehicle-treated rats were surgically castrated, while the remaining two groups were sham operated. Injections of isoproterenol or the saline vehicle were initiated two weeks after surgery was performed, to allow for adequate recovery from surgery. To reduce deaths produced by cardiac arrhythmias, the dose of isoproterenol was gradually increased from a starting dose of 0.001mg.kg^{-1} over a two week period. Isoproterenol, the final dose of 0.015 mg.kg^{-1} was selected as it resulted in no further deaths. The protocol utilising a chronic low dose of isoproterenol is a well-established model that is considered to accurately replicate the pathogenesis of human heart failure (Carll *et al* 2011).

However, spontaneous carcinogenesis did result in the deaths of some rats through the 6 month duration of the study, as expected based on the known high incidence of carcinogenesis in Sprague Dawley rats (Davis *et al* 1956).

Rats were housed in a temperature-controlled room in the Central Animal Services (CAS) of the University of the Witwatersrand for the duration of the project. The rats had

access ad libitum to both food and water, which was respectively standard rat food (supplied by EPOL, South Africa), and plain tap water. Animals were caged individually for the duration of the project.

2.2 Surgical castration

Castration was performed at approximately 10 weeks of age, as at this age the rats are nearing the end of hormonal adolescence. Although peripheral conversion of oestrogen to testosterone produces testosterone, the major source of endogenous testosterone is the testes. The castration and post-operative care was performed by trained and qualified technicians, veterinary nurses and veterinarians. Castration was performed under ketamine (80 $mg.kg^{-1}$) and xylazine (20 $mg.kg^{-1}$) general anaesthesia. Anaesthetic agents were administered via intraperitoneal injection. A small (approximately one centimetre) median incision was made at the tip of the scrotum, and the cremaster muscle exposed. The testes, caput epididymis, the cauda epididymis, the vas deferens, the testicular blood vessels and testicular fat were pulled through the incision using a blunt forceps. After clearing the fat from the bundle containing the vas deference and blood vessels, a single ligature was placed around the bundle, and the bundle was transected distal to the ligature. The testes were subsequently removed. The remaining bundle was subsequently replaced, haemostasis secured, and the cremaster muscle layer closed using a 5-0 resorbable suture. The skin was subsequently closed using a simple, interrupted suture with 3-0 nylon. Post-surgery, rats received Temgesic (Buprenorphine) (0.1 $mg.kg^{-1}$) for analgesia, and ringers lactate subcutaneously for rehydration.

2.3 Body and heart weight

Body weight was determined every week throughout the study. At the end of the study, after cardiac function had been assessed from isolated, perfused heart preparations, the atria were removed and heart weight (left and right ventricular weight) was evaluated. The right ventricular free wall as then removed from the remaining LV, and LV weight (including the septum) determined. To account for the impact of differences in absolute body weight or differences in growth on heart and LV weight, heart weight and LV weight were then indexed per 100 grams of body weight and per tibial length.

2.4 Echocardiography

Echocardiography was performed on anaesthetised rats 6 months after initiating daily isoproterenol or vehicle injections and 24 to 48 hours after the final administration of isoproterenol or its saline vehicle, using previously described methods (Norton *et al* 2002, Woodiwiss *et al* 2001). Echocardiography was performed at least 24 hours after the last dose of isoproterenol to mitigate the acute cardiovascular effects of β-AR stimulation. In this regard, isoproterenol produces β_1-AR-mediated increases in myocardial contractility, myocardial relaxation, and heart rate and β_2-AR-mediated vasodilator effects. Anaesthesia was induced via an intraperitoneal injection of 80 mg/kg ketamine and 20 mg/kg xylazine. These anaesthetic agents have contrasting effects on the cardiovascular system and this approach therefore, although not eliminating the confounding effects of anaesthesia on cardiovascular function, tends to limit these actions (Kreeger *et al* 1987). In order to perform echocardiography, the rat's chest was shaved and the rat placed in a prone position on a tray with a window exposing the chest. A prone rather than supine position was selected for imaging in order to prevent hypoxia produced by compressing the chest when pushing down on the thoracic cavity.

Echocardiography was performed with a 7.0 MHz paediatric transducer connected to an ACUSON CYPRESS portable ultrasound device (Siemens medical division, USA, Inc). In this regard, a two-dimensional image was obtained of the LV in the para-sternal short-axis view at the level of the papillary muscle. Two-dimensional targeted M-mode echocardiographic images were subsequently recorded. Images were obtained only when both the anterior and posterior LV wall endocardial surfaces were clearly visible as this allows for the determination of LV diameters and posterior wall thickness values throughout the cardiac cycle. Images were recorded for several consecutive cardiac cycles. From these images, LV end diastolic (EDD) and systolic (ESD) diameters, and posterior wall thickness (PWT) values were determined according to the American Society for Echocardiography's leading edge method (Sahn and Henry 1978). An example of an echocardiographic image and the approach to assessing LV EDD, ESD and PWT are given in Figure 2.1. A minimum of 5 consecutive, independent recordings were obtained to ensure both accuracy and precision.

To determine the degree of LV dilatation using echocardiography, LV EDD was employed as a measure of maximal LV diameters. Nevertheless, LV EDD is sensitive to variations in heart rate (a decreased heart rate allows for a greater time for LV filling and hence an increased LV EDD), volume preload (which increases LV EDD), contractility (which results in a greater LV ejection and hence a reduced LV EDD), and afterload (which results in a reduced LV ejection and hence an increased LV EDD). As LV dilatation also thins the LV wall, the extent of LV remodelling was also assessed as LV relative wall thickness which was calculated as (2 x LV end-diastolic posterior wall thickness)/LV end-diastolic diameter.

To determine LV systolic function using echocardiography, LV endocardial (FSend) and midwall (FSmid) fractional shortening were calculated. Left ventricular FSend and FSmid values were employed as indices of chamber and myocardial function respectively

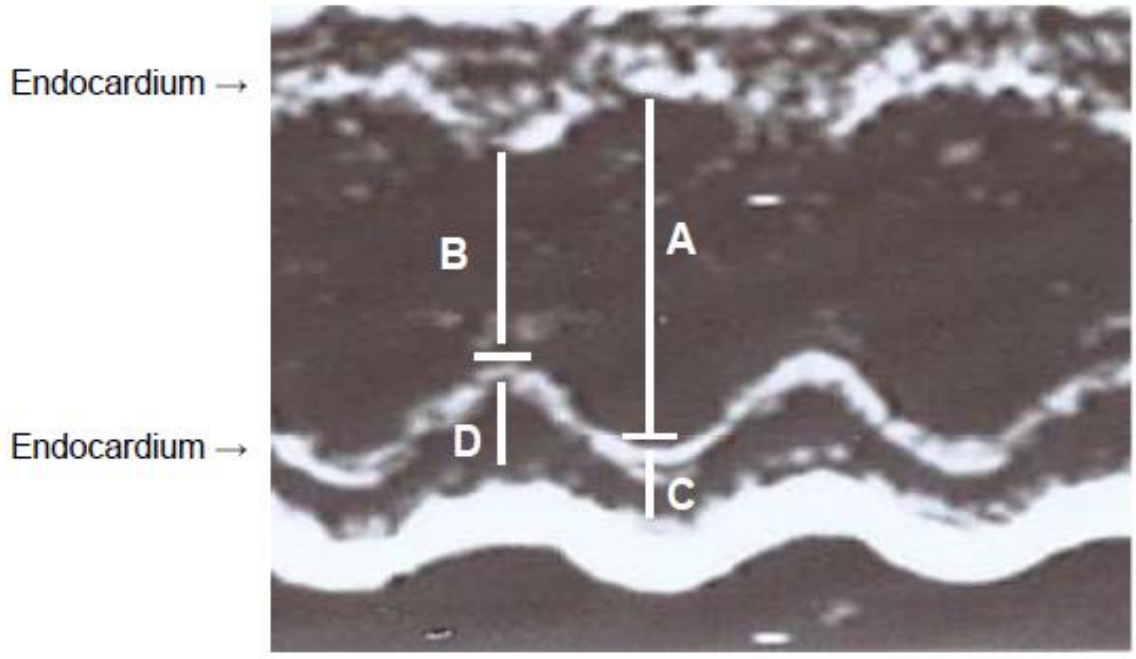


Figure 2.1 Typical two-dimensional targeted M-mode echocardiogram used to determine left ventricular dimensions. A: LV end diastolic internal diameter, B: LV end systolic internal diameter, C: LV end diastolic posterior wall thickness, D: LV end systolic posterior wall thickness, E: endocardium.

(Norton *et al* 2002, Chung *et al* 1998). Both FSend and FSmid are dependent on afterload (which reduced both) and heart rate (which increases both), as well as volume preload (Frank-Starling effect). Left ventricular FSend and FSmid were determined using the following formulae:

$$\mathbf{FS_{end} = \frac{100 \text{ X (LV EDD – LV ESD)}}{\text{LV EDD}}}$$

where:

LV EDD = left ventricular end diastolic internal diameter

LV ESD = left ventricular end systolic internal diameter

$$\mathbf{FS_{mid} = \frac{100 \text{ X ((LV EDD + LVED PWT) - (LV ESD + LV ES PWT))}}{\text{(LV EDD + LV ED PWT)}}}$$

where:

LVED PWT = left ventricular end diastolic posterior wall thickness

LVES PWT = left ventricular end systolic posterior wall thickness

For the calculation of FSmid, anterior wall thickness was assumed to be equivalent to posterior wall thickness, hence 1/2 (LV PWT) +1/2 LV anterior wall thickness was assumed to be equivalent to LV PWT.

2.5 Isolated perfused heart preparations

As LV dimensions and systolic function determined by echocardiography are influenced by loading conditions, heart rate, anaesthetic effects, neural factors and circulating concentrations of positive or negative inotropic, lusitropic and chronotropic substances, I also determined the extent of LV dilatation and differences in systolic function *ex vivo* under controlled loading conditions and heart rate using approaches previously described (Weber 1988, Norton 2002, Woodiwiss 2001). Immediately after echocardiography had been completed, a midline thoracotomy was performed under anaesthesia, the heart excised and placed in ice cold physiological perfusion solution to reduce cellular metabolic activity and to maintain viability immediately prior to perfusion (see the description of the perfusion solution in the subsequent discussion). Hearts were then mounted on a Langendorf perfusion apparatus (Figure 2.2) and retrogradely perfused via the aorta to maintain tissue viability. Using this approach an automatic pump is employed to generate a constant coronary flow. As retrograde pressure generated in the aorta closes the aortic valve, the perfusion solution moves through the coronary arteries rather than into the LV lumen.

The physiological perfusion solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$ and 10.0 glucose with a pH of 7.4 and was saturated with 95% oxygen and 5% carbon dioxide gas before being filtered through a 0.45 µm Millipore membrane. The perfusion solution was constantly gassed with a 95% oxygen and 5% carbon dioxide mixture for the duration of each study. Hearts were perfused at 12 ml/g heart weight per min. Coronary flow rate was determined from timed collections of venous effluent. The perfusion apparatus maintained the perfusate at a constant temperature and free of bubbles by first passing the perfusate through a tube surrounded by a water jacket

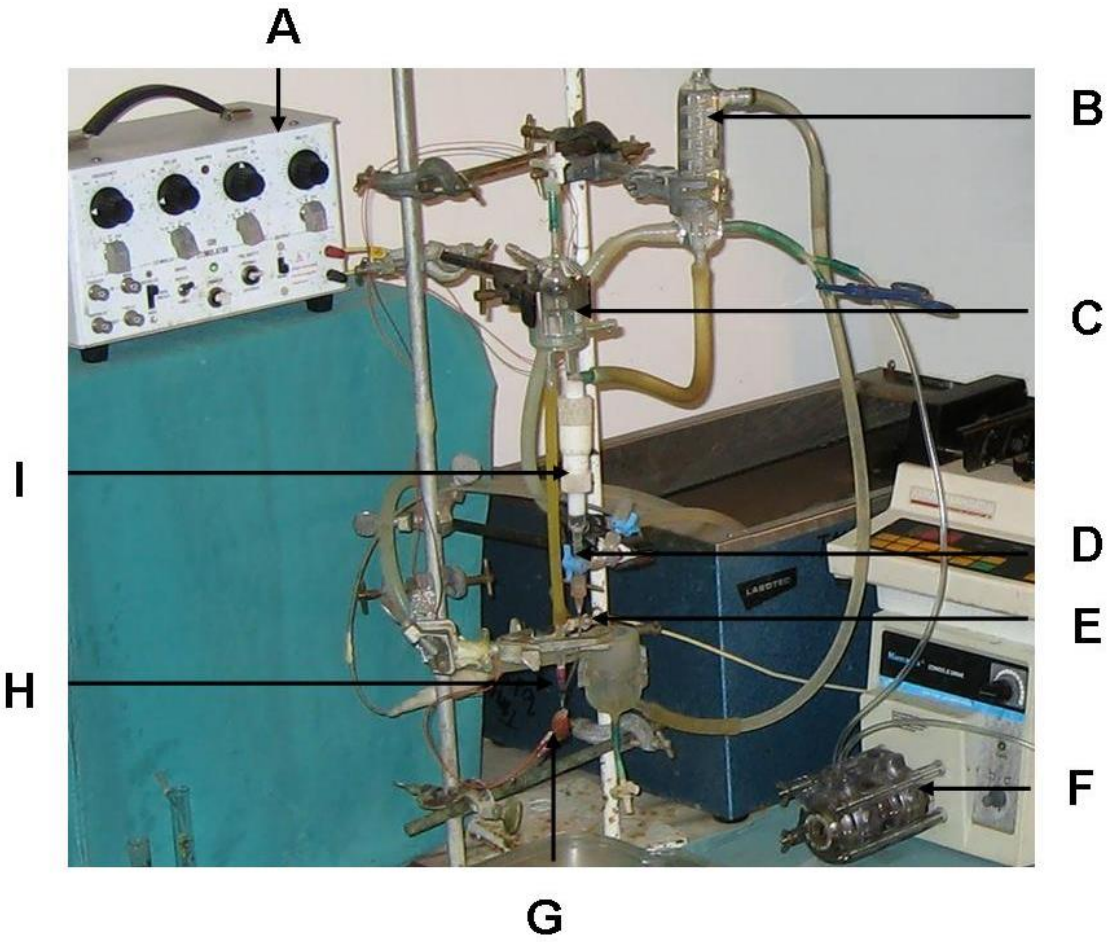


Figure 2.2. Isolated, perfused heart apparatus used in *ex vivo* experiments to assess cardiac structure and function. A, pacing device; B, heated water jacket; C, bubble trap; D, three way tap open to E, H and I; E, pressure transducer; F, peristaltic pump; G, platinum electrodes attached to the isolated heart; H, fluid filled catheter attached to latex balloon which is inserted into the left ventricular lumen; I, micromanipulator.

containing water heated to 37^{o}C, and then passing the perfusate through a bubble trap positioned proximal to the heart (Figure 2.2).

The heart was paced at 360 beats per minute via a platinum electrode placed on the right atrium and a second electrode placed on the apex of the heart. Hearts were paced using a Grass (Astro Med Inc.) model SD9 stimulator (Figure 2.2). Hearts were paced at 360 beats per minute rather than at physiological rates (approximately 400-500 beats.min^{-1}) in order to ensure that myocardial oxygen demand did not exceed supply. In this regard, although the physiological solution has a high oxygen partial pressure after saturating it with 95% oxygen; without haemoglobin, the oxygen content is below physiological levels. Nevertheless, previous studies conducted in the present laboratory have confirmed that a heart rate of 360 beats.min^{-1} is appropriate to ensure that demand induced-decreases in LV function do not occur (Umeda *et al* 2003). In this regard, at heart rates above 360 beats.min^{-1}, diastolic pressures were previously noted to begin to increase. Hearts were paced at a voltage estimated to be 10% above threshold for spontaneous excitation (Norton *et al* 2002).

To determine the extent of LV dilatation and to assess LV systolic function, LV diastolic pressures and systolic developed pressures were measured over a range of LV filling volumes. To assess LV filling volumes, a thin walled latex balloon was passed through the mitral valve and placed in the LV chamber (Figure 2.3). To assess LV diastolic pressures and systolic developed pressures, the latex balloon was coupled via a fluid-filled catheter to a Gould P50 pressure transducer (Figure 2.3). To increase LV volume the fluid-filled catheter was also coupled to a micromanipulator (Figure 2.3). The lumen of the balloon was large enough to accommodate volumes well beyond the maximum volume of the LV of either a normal rat or a rat with a dilated heart, and the pressure-volume relationship of the balloon itself only began to increase well beyond this LV volume. The volume of the balloon and catheter inserted into the ventricle was well below the ventricular volume at which diastolic

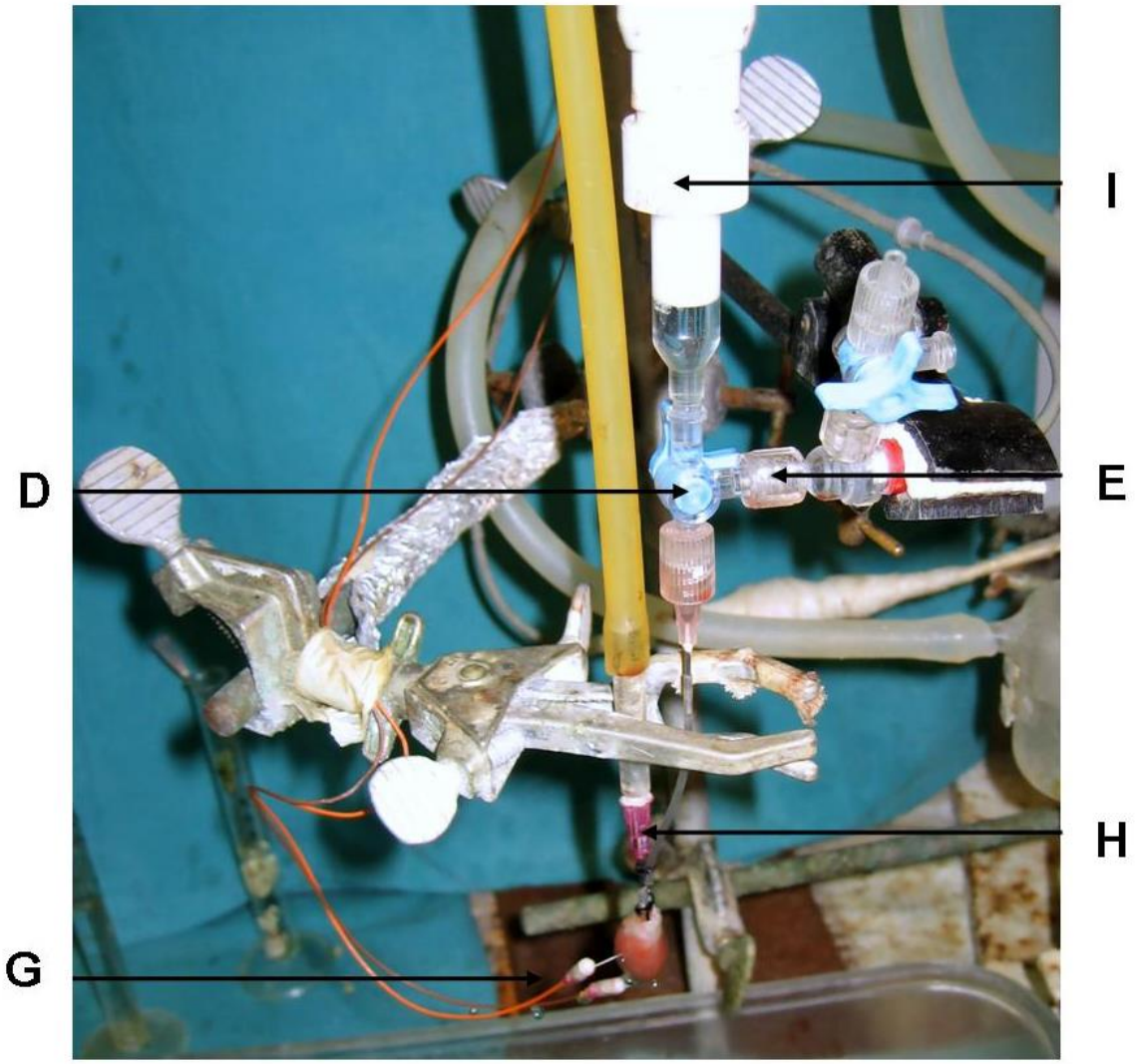


Figure 2.3. Enlargement of a portion of the isolated perfused heart apparatus depicted in figure 2.2. Clearly visible here are D, the three way tap; E, the pressure transducer; G, platinum electrodes attached to the isolated heart; H, the fluid filled catheter connected to the balloon in the left ventricular lumen and I, the micromanipulator.

pressures begin to increase, and the balloon material volume was added to balloon fluid volume to determine actual LV volume. Before placing the balloon into the LV cavity, the balloon was emptied of all excess volume by removing the micromanipulator, opening the three-way valve to atmosphere and squeezing excess fluid from the balloon via the catheter. Left ventricular pressures were determined at as many small increments in volume as were practically possible. As the micromanipulator has a Vernier scale, it allows for 0.005 to 0.01 ml increments in volume to the balloon. The micromanipulator was regularly calibrated by weighing 0.005 to 0.01 ml increments of distilled H_2O. Left ventricular developed pressures and diastolic pressures were recorded on a Hellige polygraph recorder (Figure 2.4). Calibration of the recorded LV developed pressures was performed using a mercury manometer, and LV diastolic pressures using a water-filled U-tube system designed to calibrate low pressure systems (Norton *et al* 1996). I performed calibrations for both LV developed pressure and diastolic pressure recordings after each heart preparation. As the isolated perfused heart preparation used in this dissertation is isovolumic, I assumed that LV minimum pressures (diastolic pressures) were equivalent to LV end diastolic pressure (Figure 2.4). Left ventricular developed pressures were calculated as the difference between peak LV systolic pressure and diastolic pressure (Figure 2.4).

To determine the extent of LV dilatation using a load-independent measure, LV diastolic pressure-volume relations were constructed. For statistical comparisons, the volume intercept at a diastolic pressure of 0 mm Hg (LV V_0) was identified (Badenhorst *et al* 2003a, Badenhorst *et al* 2003, Woodiwiss *et al* 2001, Norton *et al* 2002).

To assess LV systolic chamber function, LV developed pressure-volume relations were constructed and the slope of the linear portion of the relationship evaluated (LV systolic elastance-LV E) (Badenhorst *et al* 2003a, Badenhorst *et al* 2003b, Woodiwiss *et al* 2001, Norton *et al* 2002). Importantly LV E_{es} is the equivalent of LV end systolic elastance in an

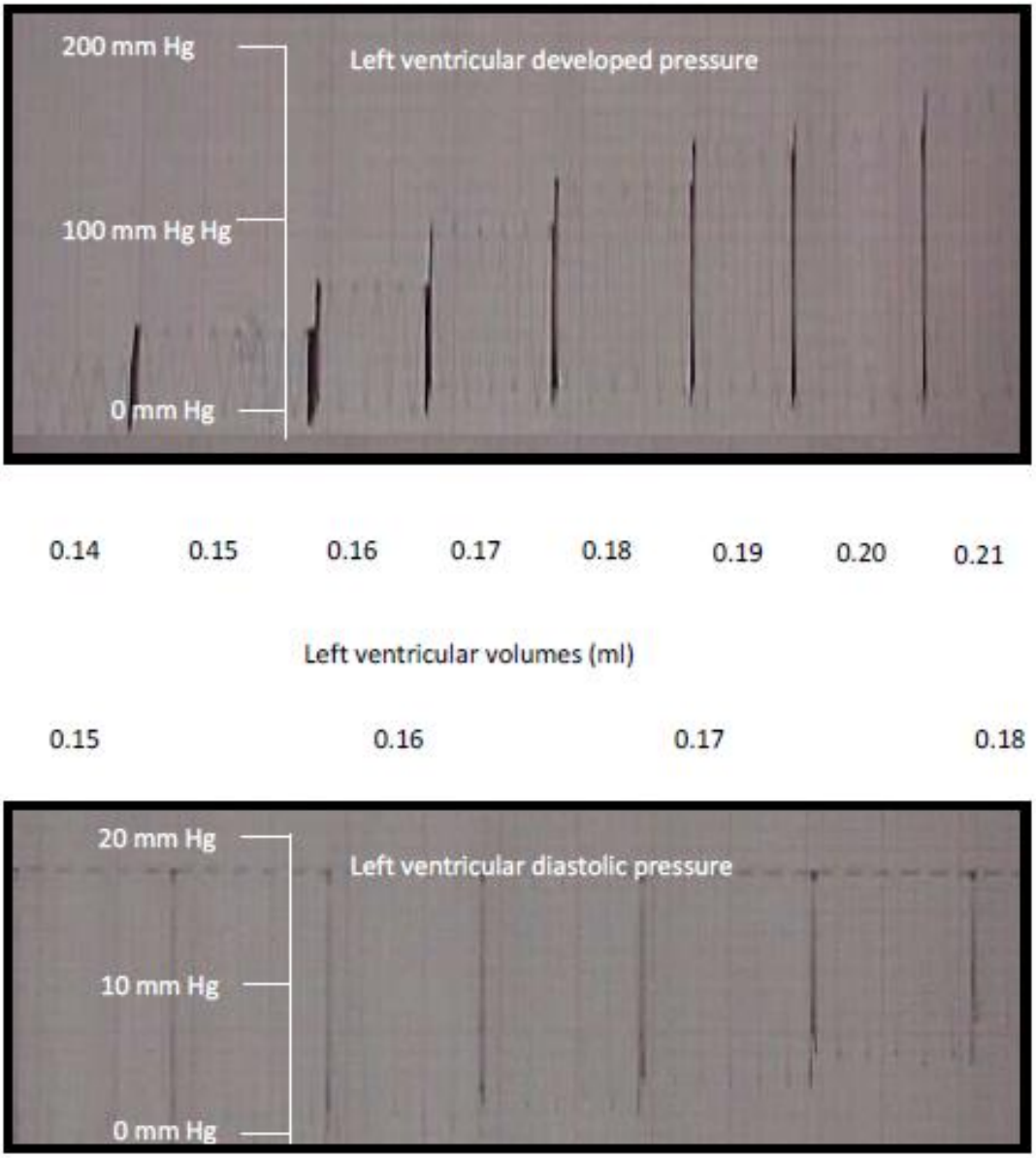


Figure 2.4. Typical recording of left ventricular developed pressure (LVDP) and diastolic developed pressure (LVEDP) pressure obtained in isolated perfused heart preparations over a range of filling volumes.

ejecting and filling LV, a well-established afterload and preload-independent measure of LV systolic chamber function (Sagawa *et al* 1981, Sagawa *et al* 1988). Data points were included in the LV systolic developed pressure-volume relationship if on linear regression analysis for individual rats, the r^2 value for the relationship was 0.95 or more. Using this approach, the first 5 LV developed pressures were included in the relationships for all rats.

To assess LV systolic intrinsic myocardial function, LV developed stress and strain relationships were constructed and the slope of the relationships evaluated (LV myocardial systolic elastance-En) (Norton *et al* 2002, Badenhorst *et al* 2003b, Veliotes *et al* 2005). By converting developed pressure and volume into stress and strain data, differences in the impact of LV geometry on systolic chamber function are accounted for and hence the slope of the stress-strain relationship can only be determined by intrinsic myocardial systolic function (Weber *et al* 1988). Although stress and strain relationships are linear along any portion of the relationships, many data points can be included for the calculation of E_n. However, to ensure that calculations of E_n were representative of the data obtained for E, E_n was calculated using only the matching ventricular developed pressure and volume data used to calculate E. Left ventricular stress and strain were calculated assuming a thick walled spherical geometry of the heart, from previously described formulae (Weber *et al* 1988, Norton *et al* 2002, Badenhorst *et al* 2003b, Veliotes *et al* 2005) as follows:

$$\textbf{Left ventricular systolic stress} = \frac{\textbf{1.36 x LV developed pressure x (LVV)}^{2/3}}{\textbf{[LVV + (0943 x LV mass)]}^{2/3} - \textbf{LVV}^{2/3}}$$

$$\textbf{Left ventricular systolic strain} = \frac{\textbf{LVV}^{1/3} + \textbf{[LVV + (0.943 x LV mass)]1/3]-1}}{\textbf{LV V}_0{}^{1/3} + \textbf{[LV V}_0 \textbf{ + (0.943 x LV mass)]}^{1/3}}$$

Where LVV is left ventricular volume and LV V_0 is the volume intercept of the LV developed pressure-volume relationship (LVV when LV developed pressure = 0 mm Hg).

2.6 Data analysis

A two-way ANOVA with a Tukey *post hoc* test was employed to determine the effect of castration or sham surgery, and 6 months of daily isoproterenol or saline administration on body weight, heart weight and LV structure and function. Linear regression analysis to determine the line of best fit for cardiac function. All values are presented as mean±SEM. Significant values were detected if $p<0.05$. The statistical software employed were SAS, version 9.3 (SAS institute Inc., Cary NC), and Prism version 5.02 (GraphPad Software Inc.).

Chapter 3

Results

3.1 Effects of castration and chronic adrenergic stimulation on body and heart weight.

Chronic isoproterenol administration to rats failed to modify either body or heart weight (Table 3.1). Castration resulted in a decrease in body weight and this effect was similar in saline- or isoproterenol-treated groups (Table 3.1). However, castration failed to influence either heart weight or LV weight in either saline- or isoproterenol-treated rats (Table 3.1). Importantly, the lack of effect of castration on heart weight or LV weight were noted irrespective of whether heart weight was expressed per 100 g body weight or per tibial length (Table 3.1).

3.2 Effects of castration on adrenergic-induced LV dilatation.

As compared to saline-treated rats, chronic isoproterenol administration resulted in an increased LV end diastolic (EDD) and end systolic (ESD) diameter (Table 3.2), a right shift in the LV diastolic pressure-volume relationship (Figure 3.1), and an increased volume intercept of the LV diastolic pressure-volume relationship (LV V_0, Figure 3.1). Castration failed to influence adrenergic-induced changes in either LV EDD or ESD (Table 3.2), the LV diastolic pressure-volume relationship (Figure 3.1), or LV V_0 (Figure 3.1).

3.3 Effects of castration on adrenergic-induced LV systolic chamber and myocardial function.

As compared to saline-treated rats, chronic isoproterenol administration failed to influence systolic chamber function as assessed *in vivo* from a load-dependent measurements

Table 3.1. Impact of castration and chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]) on body and heart weight in rats.

	Sham-operated		Castrated	
	Saline vehicle	ISO	Saline vehicle	ISO
n =	8	10	8	9
Body weight (BW) (g)	633.0 ± 28.5	623.6 ± 15.2	535.5 ± 17.2*	536.6 ± 14.1*
Heart weight (HW)(g)	1.48 ± 0.08	1.65 ± 0.08	1.47 ± 0.08	1.41 ± 0.11
LV weight (g)	1.19 ± 0.06	1.34 ± 0.14	1.16 ± 0.07	1.13 ± 0.10
HW/BW x 100	0.24 ± 0.02	0.26 ± 0.03	0.28 ± 0.02	0.26 ± 0.03
LV/BW x 100	0.19 ± 0.01	0.22 ± 0.03	0.22 ± 0.01	0.21 ± 0.02
HW/tibial length	0.30 ± 0.02	0.35 ± 0.03	0.31 ± 0.02	0.29 ± 0.02
LV/tibial length	0.24 ± 0.02	0.28 ± 0.04	0.24 ± 0.02	0.23 ± 0.02

LV, left ventricular; *$p<0.0001$ versus Sham-operated groups.

Table 3.2. Impact of castration on changes in left ventricular diameters as assessed *in vivo* (echocardiography) following chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]).

	Sham-operated		Castrated	
	Saline vehicle	ISO	Saline vehicle	ISO
n =	8	10	8	9
LV EDD (mm)	7.91 ± 0.24	9.05 ± 0.16*	7.92 ± 0.17	8.45 ± 0.14*
LV ESD (mm)	4.38 ± 0.24	5.34 ± 0.24*	4.32 ± 0.21	4.91 ± 0.12*

LV, left ventricular; EDD, end diastolic diameter; ESD, end systolic diameter; *p<0.05 versus saline-treated groups.

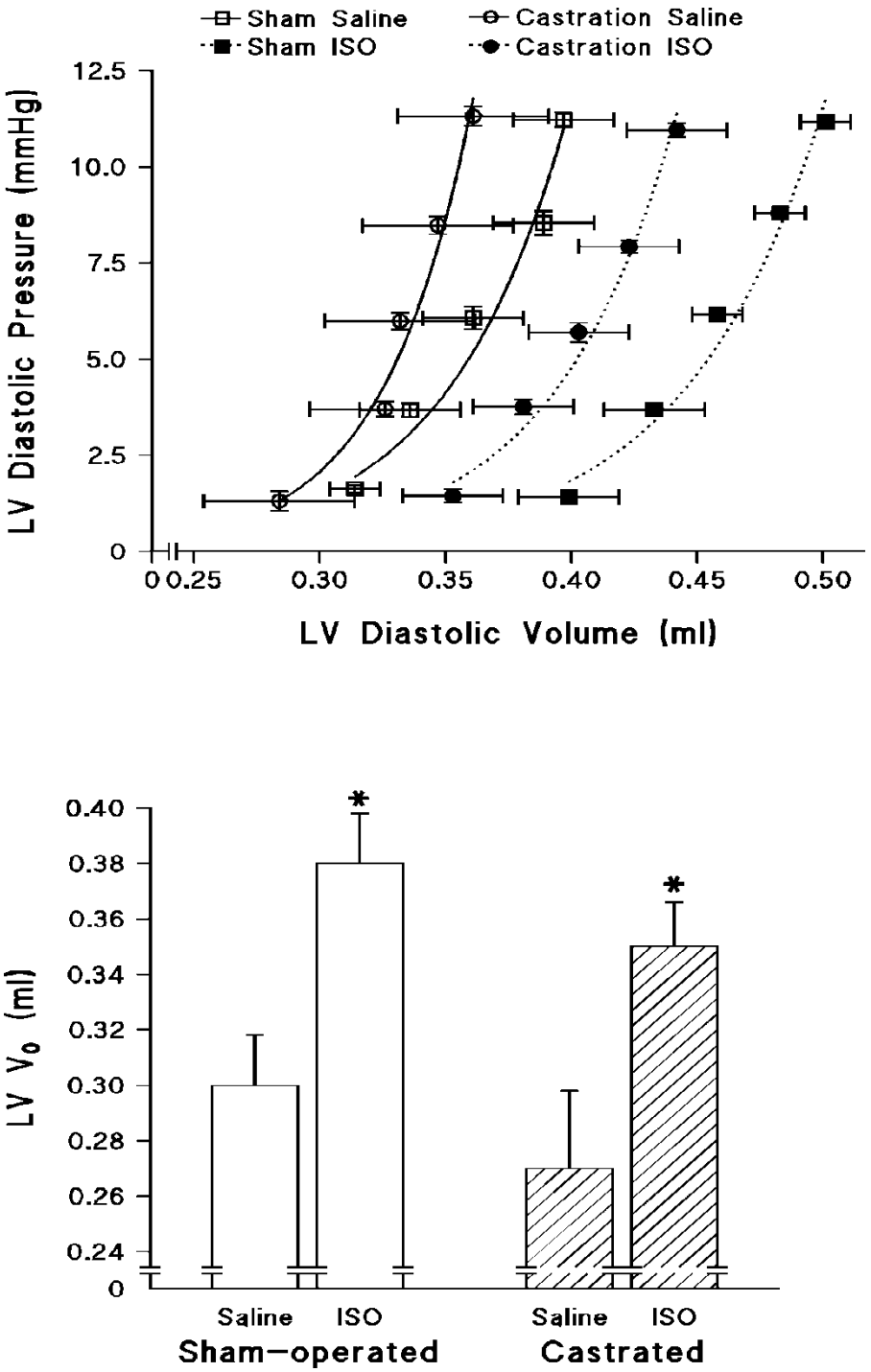


Figure 3.1. Impact of castration on changes in left ventricular (LV) diastolic pressure-volume relations following chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]). The lower panel shows the volume intercept at 0 mmHg diastolic pressure (LV V_0), an index of the extent of LV remodelling.

(LV FS_{end})(Table 3.3), as well as *ex vivo* from the LV end systolic developed pressure-volume relationship and the load-independent slope of this relationship (LV E_{es})(Figure 3.2). Moreover, chronic adrenergic stimulation did not impact on systolic myocardial function as assessed either *in vivo* from a load-dependent measurement (LV FS_{mid})(Table 3.3), or *ex vivo* from the LV end systolic developed stress-strain relationship and the load-independent slope of this relationship (LV E_n)(Figure 3.3). Castration did not influence the load-dependent measures of systolic chamber (FS_{end}) or myocardial (FS_{mid}) function (Table 3.3). Furthermore, castration did not modify the LV end systolic developed pressure-volume (Figure 3.2) or stress-strain (Figure 3.3) relationships and the load-independent slopes of these relationships (LV E_{es} and LV E_n, Figures 3.2 and 3.3).

Table 3.3. Impact of castration on changes in left ventricular systolic function as assessed *in vivo* (echocardiography) following chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]).

	Sham-operated		Castrated	
	Saline vehicle	ISO	Saline vehicle	ISO
n =	8	10	8	9
LV FS_{end} (%)	44.90 ± 1.68	41.18 ± 1.86	45.61 ± 2.07	41.81 ± 1.28
LV FS_{mid} (%)	27.13 ± 1.03	26.85 ± 2.50	27.54 ± 0.78	26.59 ± 0.95

FS_{end}, endocardial fractional shortening; FS_{mid}, midwall fractional shortening.

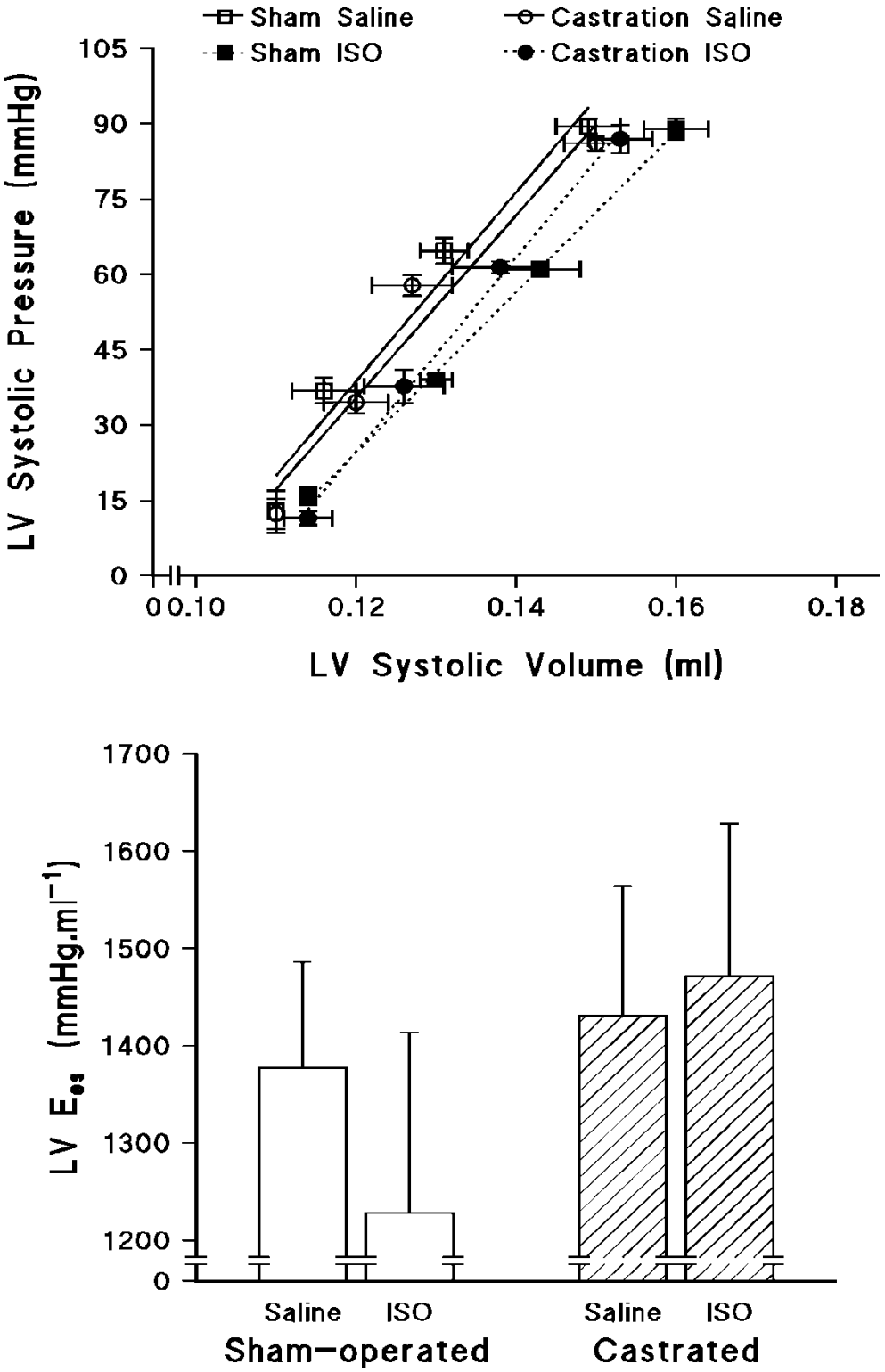


Figure 3.2. Impact of castration and chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]) on the linear portion of the LV developed pressure-volume relationship and the slope of this relationship (LV E_{es}), a load-independent index of LV systolic chamber function.

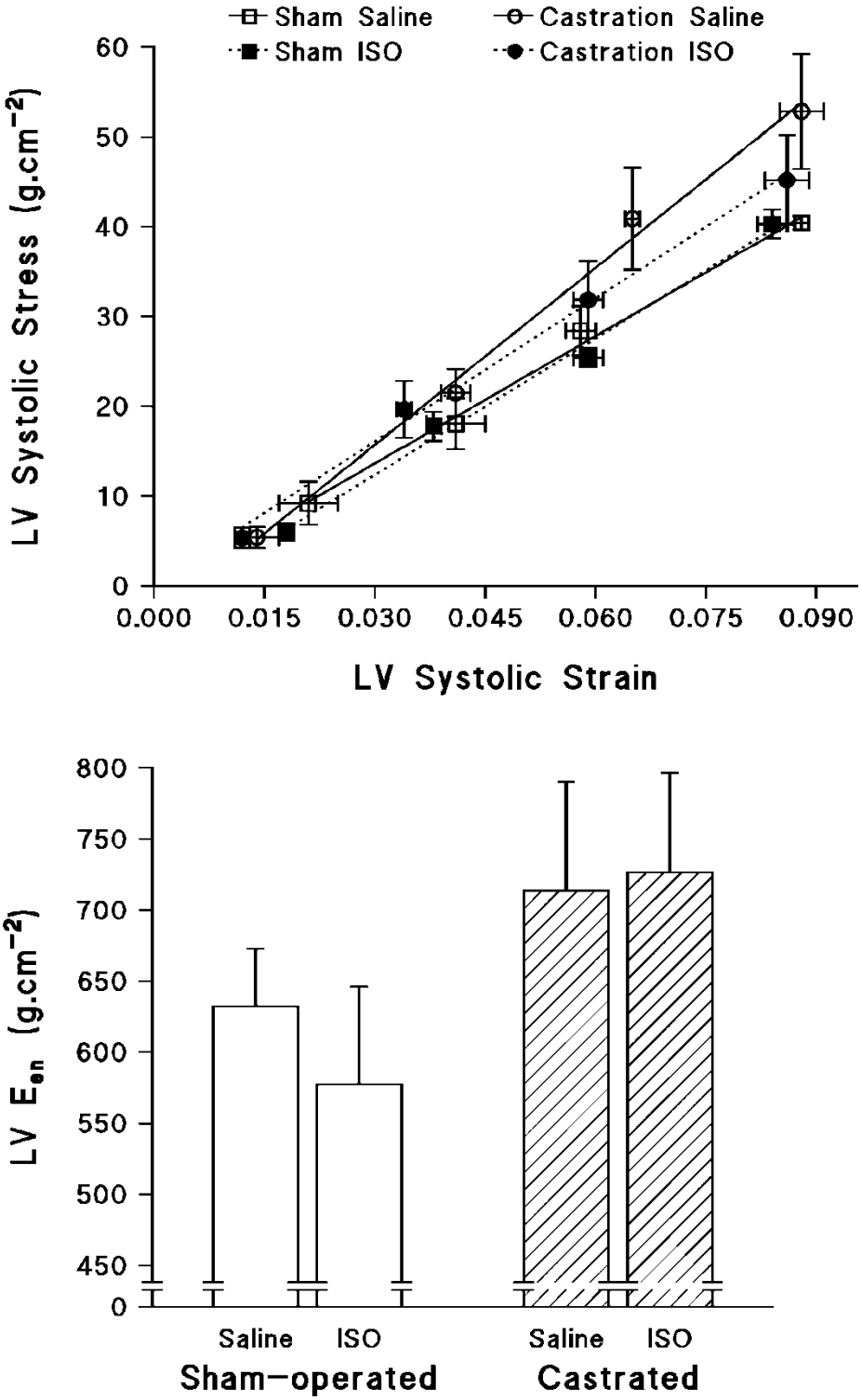


Figure 3.3. Impact of castration and chronic β-adrenergic receptor stimulation (daily isoproterenol injections for 6 months [ISO]) on the LV systolic stress-strain relationship and the slope of this relationship (LV E_n), a load-independent index of LV systolic myocardial function.

Chapter 4

Discussion

4.0 Summary of main findings

The main finding of the present study is that castration, despite producing marked decreases in body weight, had no influence on LV dilatation produced by chronic β-adrenergic stimulation in rats. Importantly, in the present study LV dilatation was assessed using a load-, heart rate-, and contractility-independent measure (LV diastolic pressure-volume relation). In addition, the lack of effect of castration on LV dilatation was noted prior to the development of decreases in LV systolic chamber or myocardial function as assessed using load-independent measurements (LV E_{es}, LV E_n).

4.1 Testosterone deficiency and LV dilatation

To the best of my knowledge the present study provides the first direct evidence that testosterone deficiency does not affect LV dilatation associated with chronic adrenergic activation. In this regard, a number of prior studies have reported on the effect of orchiectomy or castration on LV diameters in chronic cardiac disease and produced discrepant results (Gao *et al* 2003, Cavasin *et al* 2003, Montalvo *et al* 2012, Sun *et al* 2011, Kang *et al* 2012, Nahrendorf *et al* 2003, Jain *et al* 2002, Thireau *et al* 2010).

Although in some studies castration has been demonstrated to produce protective effects against increases in LV diameters in chronic mice models of cardiac disease associated with cardiac β_2-adrenergic receptor over-expression (Gao *et al* 2003, Thireau *et al* 2010) or aortic constriction (Montalvo *et al* 2012), and in a rat model of MI (Cavasin *et al* 2003), castration/orchiectomy has also been shown to augment increases in LV diameters in rat models of cardiomyocyte necrosis (Kang *et al* 2012) and, or produce no effect on LV diameters in a rat model post myocardial infarction (Nahrendorf *et al* 2003) and doxorubicin-

induced LV cardiomyopathy and dysfunction (Sun *et al* 2011). However, in all of these prior studies (Gao *et al* 2003, Cavasin *et al* 2003, Montalvo *et al* 2012, Sun *et al* 2011, Kang *et al* 2012, Nahrendorf *et al* 2003, Jain *et al* 2002, Thireau *et al* 2010), the method of assessing LV dilatation (i.e. LV diameter) is load-, heart rate- and contractile function-dependent. Hence, the diverse findings may relate to the impact of castration or the animal model studied on any one of these haemodynamic changes. In contrast, I show no effect of castration on adrenergic-induced right shifts in LV diastolic pressure-volume relations as assessed at controlled heart rates. In this regard, this measurement of adverse LV remodelling is not subject to variations in heart rate, LV contractility or LV afterload.

The ability of castration to attenuate increases in LV diameters in some studies (Gao *et al* 2003, Cavasin *et al* 2003, Montalvo *et al* 2012, Thireau *et al* 2010) may be secondary to the ability to protect against systolic chamber dysfunction in these models. Indeed, as outlined in section 1.5.1 of the introductory chapter, an improved myocardial contractility may occur subsequent to castration in these studies (Gao *et al* 2003, Cavasin *et al* 2003, Montalvo *et al* 2012) and consequently an increased systolic chamber function, could have increased ventricular ejection and hence reduced LV EDD. In contrast, an ability of orchiectomy/castration to exacerbate increases in LV diameters in other studies (Sun *et al* 2011, Kang *et al* 2012) may be secondary to worsening of systolic chamber function in the latter models. Indeed, a reduced myocardial contractility subsequent to castration in these studies (Gao *et al* 2003, Montalvo *et al* 2012) and consequently a decreased systolic chamber function, could have decreased ventricular ejection and hence enhanced LV EDD. However, unlike these prior studies (Gao *et al* 2003, Cavasin *et al* 2003, Montalvo *et al* 2012, Sun *et al* 2011, Kang *et al* 2012, Jain *et al* 2002, Thireau *et al* 2010) where LV EDD changes subsequent to castration may have been secondary to modifications in systolic chamber function, I report on a lack of effect of castration on the LV diastolic pressure-volume

relation, prior to the development of systolic chamber dysfunction as assessed from LV E_{es} and LV E_n. Hence, the present study provides strong evidence to suggest that testosterone deficiency has little effect on the primary remodelling process responsible for LV dilatation in cardiac disease.

One possibility to explain the lack of effect of castration on LV dilatation in the present study is that although castration resulted in marked decreases in body weight this failed to translate into decreases in heart weight. In this regard, LV dilatation may be associated with increases in LV weight and hence decreases in heart weight may be required to return LV diameters to normal. Indeed, Gao *et al* (2003) and Thireau *et al* (2010) reported on a beneficial effect of castration on adrenergic-induced LV dilatation in association with a decrease in heart weight indexed for body weight. Moreover, in a number of studies that failed to show a beneficial effect of castration on LV dilatation in animal models of LV dilatation associated with cardiomyocyte necrosis (Kang *et al* 2012), MI (Nahrendorf *et al* 2003) or doxorubicin-induced myocardial damage (Sun *et al* 2011), castration also failed to reduce heart weight. However, despite a beneficial effect of castration on LV diameters in a model of aortic constriction, castration failed to decrease heart weight (Montalvo *et al* 2012). Hence, although there is a possibility that reductions in heart weight are required for castration to reduce LV cavity dimensions, this does not appear to be the only factor involved.

A caveat of the present study is that I did not show that LV dilatation ultimately translates into a reduced LV systolic chamber function following chronic β-AR activation. Hence, it may be argued that the neutral effect of testosterone deficiency on LV dilatation observed in the present study, does not preclude the possibility that testosterone replacement therapy in heart failure does not ultimately worsen LV dilatation associated with systolic chamber dysfunction. However, our research group has previously demonstrated that with

daily administration of modestly higher doses of isoproterenol than that given in the present study, that LV dilatation is indeed associated with the development of LV systolic chamber dysfunction (a reduced LV end systolic elastance) (Woodiwiss *et al* 2001, Osadchii *et al* 2007, Booysen *et al* 2012). Moreover, the association between LV dilatation and LV systolic chamber dysfunction in these prior studies was noted to be a key mechanism responsible for the reduced systolic chamber function (Osadchii *et al* 2007, Booysen *et al* 2012). Indeed, LV systolic chamber dysfunction in these prior studies (Osadchii *et al* 2007, Booysen *et al* 2012) occurred in the absence of changes in LV E_n (myocardial systolic dysfunction). Hence, it is likely that the LV dilatation evaluated in the present study does indeed have important pathophysiological implications.

4.2 Testosterone deficiency and LV systolic chamber function.

An important consideration is that the testosterone deficiency that is likely to have been produced by castration in the present study failed to influence either LV systolic chamber function or LV intrinsic myocardial systolic function in either normal rats or in rats exposed to chronic adrenergic activation. To this effect, the previous studies investigating the effect of testosterone withdrawal or replacement on cardiac function show discrepant results (Cavasin *et al* 2003, Montalvo *et al* 2012, Sun *et al* 2011, Kang *et al* 2012, Jain *et al* 2002, Sebag et al 2011, Curl *et al* 2008, Golden *et al* 2003). What could explain these discrepancies?

One possible explanation for the discrepancies between studies evaluating the impact of castrations on cardiac systolic function, is the differences in the models studied. In this regard, Sun *et al* (2011) and Kang *et al* (2012) made use of acute models of myocardial toxicity and in both instances castration caused a greater degree of cardiac systolic

dysfunction, while post-castration treatment with testosterone ameliorated the effect of castration. In contrast, Gao *et al* (2003) and Thireau *et al* (2010) employed models of adrenergic-induced cardiac dysfunction and Montalvo *et al* (2012) studied an animal model of aortic constriction and demonstrated beneficial effects of castration. Hence, it is possible that castration has different effects on the adverse actions of acute (Montalvo *et al* 2012) as opposed to more chronic (Gao *et al* 2003, Thireau *et al* 2010) myocardial changes. However, studies evaluating the impact of castration on cardiac function post-MI, where the pathology may be considered to be a combination of acute and chronic myocardial damage has been shown to produce both beneficial (Cavasin *et al* 2006) and neutral (Nahrendorf *et al* 2003) effects. Therefore it is difficult to ascribe the differential effects of castration on systolic cardiac function in animal models of cardiac pathology on the different models of disease studied.

4.3 Effects of castration of cardiac weight

An important question which arises from the present study is why castration resulted in reductions in heart weight in some but not other studies (including the present study)? In this regard, the timing of castration may be considered as being important. In one study where heart weight was reduced by castration, castration was performed at three weeks of age, a point clearly before the onset of adolescence (Thireau *et al* 2010). Moreover, Montalvo *et al* (2012) failed to show an effect on heart weight when mice were castrated at one year of age, an age well into sexual maturity. In contrast however, in a study where castration resulted in a decrease in heart weight, castration was only performed at 3 months of age (Gao et al 2003), an age that is well past the onset of adolescence in murine models. Furthermore, Cavasin *et al* (2006) orchidectomized mice at four weeks of age, and Nahrendorf *et al* (2003)

similarly orchidectomized animals at a very young age, and neither group of authors showed effects on heart weight. Moreover, in the present study I similarly castrated pre-pubescent rats and despite a marked effect on body weight, failed to show an impact of castration on heart weight (Hodson *et al* 2014). Hence, further studies are required to attempt to explain discrepancies in the impact of castration on heart weight.

4.4 Potential clinical implications

The clinical importance of the present study warrants consideration. Although testosterone deficiency in heart failure is independently associated with a poor prognosis (Jankowska *et al* 2006, Güder *et al* 2010, Kontoleon *et al* 2003), no obvious or only minor effects on the LV are noted when testosterone is administered up to 12 months in patients with heart failure (Malkin *et al* 2006, Caminiti *et al* 2009). However, longer periods of testosterone replacement therapy in patients with heart failure than that already assessed (1-12 months) (Malkin *et al* 2006, Chung *et al* 2007) may have adverse effects on LV dilatation. Thus, whether the benefits of testosterone replacement therapy in patients with heart failure on skeletal muscle strength, lean muscle mass, endurance, and neuromuscular and baroreceptor reflexes (Malkin *et al* 2010) could be exploited for prolonged periods is unknown. The present study provides some confidence that testosterone deficiency has no protective benefits on LV dilatation and hence that testosterone replacement therapy raised to normal levels is unlikely to produce adverse consequences to the primary LV remodelling process in chronic cardiac disease.

There is an important caveat to the present study, with major clinical implications, that requires consideration. Orchiectomy/castration has been demonstrated to produce protective effects against LV systolic function in mice models of cardiac disease associated

with cardiac β_2-adrenergic receptor over-expression (Gao *et al* 2003) or aortic constriction (Montalvo *et al* 2012). Thus, there is still a possibility that testosterone replacement therapy may produce adverse effects on cardiac function in heart failure associated with pressure-overload states or when inadequate adrenergic receptor blockade occurs, but most likely in heart failure with an intrinsic cardiomyopathy (Sun *et al* 2011, Kang *et al* 2012). Further clinical studies are therefore required to assess the impact of testosterone replacement therapy on pressure-overload-induced cardiac failure or in patients unable to tolerate adequate doses of β-adrenergic receptor blocking agents.

4.5 Does the present study contribute toward our current understanding of gender differences in cardiac structure and function?

Although not an aim of the present study it is worth considering whether the present study contributes toward our present understanding of the gender differences which exist in cardiac structure and function in either healthy individuals or in cardiac disease. As discussed in chapter 1, there is considerable evidence to suggest that males have a more eccentric LV than females and that this translates into reductions in LV systolic chamber function (see sections 1.3 and 1.4). Moreover, in heart failure or cardiac disease, males are more likely to develop a greater degree of LV dilatation than females and this translates into a greater chance of developing a reduction in LV systolic chamber function in males as compared to females (see sections 1.3 and 1.4). In this regard, it is well recognised that LV dilatation is produced by neurohumoral activation in heart failure (see section 1.2.5). Hence, one possible factor which may account for gender differences in LV dimensions and systolic chamber dysfunction is through an interaction between testosterone and adverse sympathetic effects on the heart. However, as indicated in section 1.5.2 of chapter 1, studies investigating the

relationship between gender and adrenergic stimulation in pathology, report potentially beneficial or detrimental actions of male gender and testosterone depending on the pathology involved. Does the current study support or oppose these data?

Myocardial adrenergic down-regulation may have adverse effects in chronic heart failure. Indeed, after four weeks of volume overload induced via an arterio-venous fistula, in which eccentric LV remodelling was clearly detected in male but not female rats, a reduction of β_1-adrenergic receptor mRNA expression was noted in male rats as compared to their controls, whilst female rats had no such reduction (Dent *et al* 2010, Dent *et al* 2011). Furthermore, β_2-adrenergic receptor mRNA expression was increased in female hearts relative to control animals, but not in males and β_1 and β_2-adrenergic receptor density and concentration was reduced in male hearts, whilst they were conversely increased in female hearts (Dent *et al* 2011). Whether these myocardial adrenergic changes in male rats represent a compensatory response to protect the myocardium against excessive adrenergic stimulation, or whether they contribute toward LV dilatation and reductions in systolic chamber function is uncertain. In this regard the present study suggests that if these changes are attributed to an interaction between sympathetic stimulation and testosterone (Dent *et al* 2010, Dent *et al* 2011), that they are unlikely to contribute toward LV dilatation and systolic chamber dysfunction.

In studies involving transgenic mice with β_2-adrenergic receptor overexpression, male mice have been noted to develop increased LV diameters and a reduced LV EF relative to the female mice (Gao *et al* 2003, Thireau *et al* 2010). Furthermore, gonadectomy in male mice reduced LV diameters and increased LV EF (Gao *et al* 2003, Thireau *et al* 2010). These data suggest that at least in the context of excessive β_2-adrenergic receptor over-activation, testosterone interacts with the adrenergic stimulus to enhance the adverse effects of adrenergic activation on adverse LV remodelling and LV EF. However, the present results

challenge this hypothesis and suggest that if an interaction between testosterone and sympathetic stimulation contributes toward adverse LV remodelling, that this effect must be secondary to the deleterious effects of adrenergic actions on myocardial systolic function, which will cause cardiac dilatation secondary to reductions in chamber contraction.

4.6 Limitations of the present study

The potential limitations of the present study warrant consideration. First, although I demonstrated that castration was unable to modify LV dilatation mediated by chronic excess adrenergic receptor activation, I did not assess the impact on the mechanisms that contribute toward adrenergic-induced LV dilatation. In this regard, the current findings could have been further supported by measurements of myocardial collagen characteristics (Gunja-Smith *et al* 1996, Badenhorst *et al* 2003a, Lindsey *et al* 2003, Weber *et al* 1988), and apoptosis (Communal *et al* 1998, Fan *et al* 2006, Garg *et al* 2005). Nevertheless, even if castration had modified any of the basic mechanisms involved in mediating cardiac dilatation, these changes clearly failed to translate into changes in the adverse LV diastolic remodelling process. Second, as already acknowledged, I did not assess the impact of castration on a model of adrenergic-induced LV dilatation with systolic chamber dysfunction. However, as explained in the aforementioned discussion, this allowed for the assessment of the impact of castration on LV dilatation prior to systolic chamber decompensation. Hence, an interpretation of the data without the confounding influence of systolic chamber dysfunction on LV dilatation was possible. Third, LV diastolic pressure-volume relations were determined in an isovolumic cardiac preparation, which does not allow for separation of early and late diastolic phases of the cardiac cycle. In this regard the factors which determine early and late diastolic pressure-volume relations are likely to differ (Gibert and Glantz 1989). Fourth, the extent to which

castration reduced circulating testosterone concentrations was not assessed. Nevertheless, although peripheral conversion of oestrogen to testosterone produces testosterone, the major source of endogenous testosterone is the testes. Evidence in favour of marked testosterone deficiency following castration was the presence of an almost 100g difference in body weight between sham-operated and castrated rats at the end of the study.

4.7 Conclusions

In conclusion, the present study shows that castration has no effect on right shifts in LV diastolic pressure-volume relations produced by chronic β-adrenergic receptor stimulation. The lack of effect of castration on LV diastolic pressure-volume relations was noted prior to the development of decreases in LV systolic function as assessed using load-independent measurements. Hence, the present results provide the first direct evidence to suggest that testosterone deficiency in chronic cardiac disease has no effect on the primary mechanisms responsible for LV dilatation. Thus, chronic testosterone replacement therapy in heart failure may not exacerbate adverse LV remodelling. In support of the present dissertation, the study has been published (Hodson *et al* 2014).

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/25/04

APPLICANT: Mr B Hodson

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **The effect castration or testosterone receptor blockade on β-adreneric induced left ventriclar pump dysfunction in male rats**

Number and Species

128 Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 04.05.2010. This approval remains valid until 04.05.2012

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

- Researcher lists himself as either the investigator or a co worker.
- The researcher has not indicated that flutamide is a testosterone receptor blocker. The researcher should clarify this for the committee.
- Saline needs to given for 2 weeks prior to starting the isoproterenol injections to habituate rats to the animal handling and the procedure.

Signed: _____ Date: 20/05/2010
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 20/05/2010
(Registered Veterinarian)

cc: Supervisor:
Director: