The Estrous Cycle Modulates Contractile Function and Ca^{2+} Homeostasis In Isolated Mouse Ventricular Myocytes

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
July 2012

DALHOUSIE UNIVERSITY

DEPARTMENT OF PHARMACOLOGY

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Dated: July 9, 2012

Supervisor: _____

Readers: _____

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DALHOUSIE UNIVERSITY

DATE: July 9, 2012

AUTHOR: Jennifer K. MacDonald

TITLE: The Estrous Cycle Modulates Contractile Function and Ca^{2+} Homeostasis In Isolated Mouse Ventricular Myocytes

DEPARTMENT OR SCHOOL: Department of Pharmacology

DEGREE: MSc CONVOCATION: October YEAR: 2012

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Signature of Author

Table of Contents

List of Tables

List of Figures

Abstract

This study investigated the effect of the mouse estrous cycle on myocyte contractile function. Female mice displayed irregular estrous cycles unless induced to cycle though exposure to bedding collected from cages housing male mice. Fractional shortening and Ca^{2+} transient amplitudes were significantly larger in myocytes isolated from mice in estrus. The effect of the estrous cycle was preserved even when cells were paced at a more physiological frequency and in the presence of β-adrenergic stimulation. Myofilament Ca^{2+} sensitivity was also modified by the estrous cycle, as myofilaments isolated from the hearts of mice in estrus were least sensitive to Ca^{2+}. However, acute application of either 17β-estradiol or the G protein-coupled estrogen receptor (GPER) agonist, G-1, had no effect on contractions or Ca^{2+} transients, regardless of the estrous stage. Thus, physiological fluctuations in sex hormone levels modify myocyte contractions, Ca^{2+} release, and myofilament Ca^{2+} sensitivity.

List of Abbreviations and Symbols Used

°C	Degree Celsius
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
AC	Adenylyl cyclase
ANOVA	Analysis of variance
APD	Action potential duration
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase enzyme
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CICR	Calcium induced calcium release
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	17β-estradiol
EC	Excitation-contraction
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERK	Extracellular signal-regulated kinase
FSH	Follicle stimulating hormone
GPER	G protein-coupled estrogen receptor
GPR30	Previous name for GPER
h	Hours
Hz	Hertz
ISO	Isoproterenol
$I_{Ca,L}$	L-type Ca2+ current
$I_{K,slow}$	Ultra-rapid delayed rectifier K^+ current
I_{to}	Transient outward K^+ current
K^+	Potassium ion
LH	Luteinizing hormone
kg	Kilogram

mA	Milliamp
mg	Milligram
min	Minute
mins	Minutes
mos	Months
ml	Milliliter
mM	Millimolar
mV	Millivolt
nm	Nanometer
nM	Nanomolar
Na^+	Sodium Ion
NCX	Na^+/Ca^{2+} exchanger
O_2	Oxygen
OVX	Ovariectomized
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PTI	Photon Technology International
RyR	Ryanodine receptor
RM ANOVA	Repeated measures analysis of variance
sec	Second
SEM	Standard error mean
msec	Millisecond
secs	Seconds
SERCA	Sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
SVT	Paroxysmal supraventricular tachycardia
β_1-AR	β_1-andrenergic receptor
μg	Microgram
μm	Micrometer
μM	Micromolar

Acknowledgements

First and foremost, I would like to acknowledge my supervisor, Dr. Susan Howlett, for her advice, encouragement, and guidance throughout this project. Thank you for providing me with such a positive working environment to develop as a scientist and to gain important life skills. It has been a privilege to learn from such a passionate and highly motivated scientist. I will always cherish the wonderful memories I have from the years in your lab.

I would also like to thank Peter Nicholl and Dr. Jie-quan Zhu for your excellent training, technical advice, and helpful troubleshooting throughout this project. To both of you, thank you for your willingness to assist me with any questions that I encountered throughout my research. Thank you also for your friendship and the many laughs we've shared along the way.

Additionally, I would like to thank my colleagues, Leo Fares, Randi Parks, and Jeanne Egar. You have been exceptional friends. Thank you for your ongoing support both in and out of the lab. I will truly miss our times spent together. I know you will all excel in the careers you choose to pursue in the future. As well, I would like to thank Matthew Ernst for his unwavering support and encouragement throughout this process. You have always been there for me with patience and understanding.

Finally, I would like to thank the staff of Department of Pharmacology, Luisa Vaughan, Sandi Leaf, Cheryl Bailey, and Janet Murphy for always assisting me with administrative tasks and answering all of my questions. Your assistance has been invaluable over the past two years.

Chapter 1: Introduction

I. Overview

i. Sex Differences in Cardiovascular Disease

It is well established that there are sex differences in the prevalence of cardiovascular disease and overall cardiovascular pathophysiology (Czubryt et al., 2006). Even as early as 1913, it was observed that men had much higher mortality than women when diagnosed with hypertensive cardiovascular disease (Janeway, 1913). This is consistent with the results of more recent clinical studies. The prevalence of cardiovascular disease in pre-menopausal women is much lower than in age-matched men, however, this female advantage declines following menopause (Barrett-Connor and Bush, 1991). It has been suggested that estrogen may have a protective effect on the heart, as bilateral oophorectomy has been shown to increase the risk of adverse cardiovascular events in females (Hayward et al., 2000). The reasons underlying the protective effects of estrogen on the heart are poorly understood. However, cardiac myocytes have been shown to contain the nuclear estrogen receptors α and β (Stumpf et al., 1977). Therefore, it is likely that the protective effects of estrogen on the myocardium may be mediated, at least in part, at the level of the individual cardiomyocyte.

Along with the protective effects estrogen may have on the heart itself, it is known that estrogen attenuates the development of atherosclerosis and improves overall vascular function (Vaccarino et al., 1999). Clinically, women present much later in life with their first myocardial infarction when compared to men (Vaccarino et al., 1999). It is thought that estrogen protects the vasculature through increases

in eNOS which leads to vasodilation and regeneration of the endothelium of the vasculature (Meyer and Barton, 2009).

Although estrogen is implicated in the protective effects of female sex on the cardiovascular system, estrogen levels are not constant but fluctuate during the menstrual cycle (Lentz, 2012). Therefore, the female menstrual cycle must be taken into account when investigating the effects of estrogen on the heart. Indeed, various cardiovascular parameters are known to fluctuate with the human menstrual cycle. Several studies have investigated the effect of the menstrual cycle on resting heart rate (Hassan et al., 1990; Kaplan et al., 1990; Kelleher et al., 1986; Manhem and Jern, 1994). These studies have shown that resting heart rate is elevated when estrogen levels are low during the luteal phase of the menstrual cycle and that heart rate decreases during the follicular phase when estrogen levels rise. Low estradiol levels present during the luteal phase of the menstrual cycle are also associated with an increased prevalence and increased duration of paroxysmal supraventricular tachycardia (SVT) in pre-menopausal women with no history of cardiovascular abnormalities (Rosano et al., 1996). Taken together, these results suggest that fluctuations in hormone levels associated with the menstrual cycle can affect cardiovascular function. Experimental animals, such as mice, exhibit cyclical alterations in hormone levels known as the estrous cycle (Butcher et al., 1974). Whether the estrous cycle affects cardiovascular function is not yet known. The overall goal of this thesis is to determine whether the estrous cycle modulates myocyte contractile function in sexually-mature female mice.

II. Myocyte Contractile Function

i. The Ventricular Myocyte

The heart is made up of individual contractile cardiac muscle cells referred to as cardiomyocytes . Approximately 75% of the mass of the ventricles is cardiomyocytes (Loscalzo, 2012). Ventricular myocytes range from 60-140 μm in length and 17-25 μm in diameter (Loscalzo, 2012). They are typically bi-nucleated and have a striated appearance due to the presence of the myofilaments. The following section provides a detailed summary of how an electrical impulse, referred to as the action potential, initiates cardiomyocyte contraction mediated by the myofilaments through activation of the excitation-contraction coupling pathway.

ii. Overview of the Ventricular Action Potential and Excitation Contraction Coupling

At rest, the intracellular concentrations of Na^+, K^+, and Ca^{2+} in ventricular myocytes are 10 mM, 135 mM, and 0.1 mM, respectively (Ten Eick et al., 1981). These values differ significantly from the extracellular concentrations, which are 145 mM, 4 mM, and 2 mM, respectively. This electrochemical gradient gives rise to a resting membrane potential of approximately -80 mV (Levy, 2007). Figure 1.1A shows a schematic of a human ventricular myocyte action potential. The various phases are characterized by ion movement across the cell membrane. At rest, the sarcolemma is permeable to K^+, but not to Na^+ and Ca^{2+}. When the membrane is depolarized to approximately -65 mV, the large electrochemical gradient for Na^+ results in a rapid influx of Na^+ which gives rise to the up-stroke of the action potential, or phase 0 (Levy, 2007). Na^+ channels quickly inactivate, and a brief

period of repolarization is observed as an efflux of K^+ through the transient outward current (I_{to}). This is seen as the notch at the top of the action potential (phase 1). The plateau of the action potential, or phase 2, is characterized by the influx of Ca^{2+} through the L-type and T-type Ca^{2+} channels (Bean, 1985). L-type Ca^{2+} channels are much more abundant in the heart than T-type Ca^{2+} channels. Further extrusion of K^+ from the cell causes repolarization, which is known as phase 3 of the action potential. The Na^+/K^+ ATPase pumps 3 Na^+ out in exchange for 2 K^+ in, in order to return ion concentrations to resting values (Apell and Karlish, 2001). Figure 1.1B shows a schematic of a mouse ventricular action potential. Phases 1, 2, and 3 of the action potential are not as well defined as those seen in the human action potential. This quick repolarization is due to the rapid activation of numerous repolarizing K^+ currents, mainly a fast I_{to} (Knollmann et al., 2007).

Contraction of the heart is mediated by a process known as the excitation contraction (EC) coupling pathway (Bers, 2002). Figure 1.2 provides an overview of the EC coupling pathway. EC coupling is the process by which the electrical stimulus, or action potential, is converted into a mechanical response. In the EC coupling pathway, an action potential propagating along the sarcolemma extends to the interior of the cell via T-tubules. This depolarization of the membrane opens voltage-sensitive L-type Ca^{2+} channels, which results in an influx of Ca^{2+} into the cytosol (Bers, 2002). This incoming Ca^{2+} gives rise to the L-type Ca^{2+} current ($I_{Ca,L}$). $I_{Ca,L}$ interacts with ryanodine receptors (RyR) located on the sarcoplasmic reticulum (SR) and causes their activation and opening (Bers, 2002). This subsequently leads to Ca^{2+}-induced Ca^{2+} release (CICR) from the SR, which gives rise to the Ca^{2+}

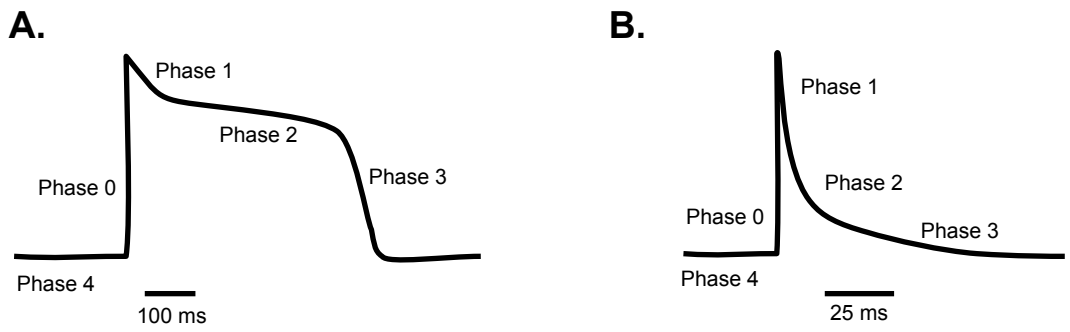


Figure 1.1. Schematic of the human and mouse ventricular action potentials. **A:** Human ventricular myocyte action potential. Depolarization of the membrane to approximately -65 mV results in a rapid influx of Na^+ which gives rise to the up-stroke of the action potential (phase 0). Na^+ channels quickly inactivate, and a brief period of repolarization is observed as an efflux of K^+ through the transient outward current (I_{to}). This is seen as the notch at the top of the action potential (phase 1). The plateau is characterized by the influx of Ca^{2+} through the L-type Ca^{2+} channel (phase 2). Further extrusion of K^+ from the cell mediates repolarization (phase 3). **B:** Mouse ventricular action potential. Ion movement is similar to that seen in human cardiomyocytes. Phases 1, 2, and 3 of the action potential are not as well defined. The rapid repolarization is due mainly to a fast I_{to}.

transient. The released Ca^{2+} interacts with the myofilaments and causes a contraction. Three factors are key determinants of the force of contraction: Ca^{2+} transient amplitude, Ca^{2+} transient duration, and myofilament Ca^{2+} sensitivity (Bers, 2002). Cardiomyocyte relaxation occurs when intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is lowered. Reduction in $[Ca^{2+}]_i$ occurs in several ways. Ca^{2+} is sequestered back into the SR via the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Ca^{2+} is extruded from the cell via the Na^+/Ca^{2+} exchanger (NCX). SERCA and NCX are responsible for approximately 92% and 7% of Ca^{2+} removal from the cytosol, respectively, in rodent cardiomyocytes (Bassani et al., 1994; Hove-Madsen and Bers, 1993; Li et al., 1998). Phospholamban (PLB) is an SR protein that controls the rate at which Ca^{2+} is taken back up into the SR through SERCA activation (Bers, 2002). In its un-phosphorylated state, it suppresses SERCA activity. The sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter are responsible for the removal of the remaining 1% of Ca^{2+} in the rat cardiomyocyte (Bassani et al., 1994).

Ca^{2+} cycling is a tightly regulated process within the heart (Bers, 2002). Disruptions in Ca^{2+} movement within the cardiomyocyte are associated with the development of various cardiovascular diseases (Katz and Reuter, 1979). For example, Ca^{2+} overload within the heart is a key trigger for ventricular arrhythmias and is also associated with chronic heart failure (Frommeyer et al., 2012). Previous studies have suggested that the cardioprotective effects of estrogen may be due to alterations in Ca^{2+} movement within the EC coupling pathway (Curl et al., 2001; Farrell et al., 2010; Leblanc et al., 1998). Ca^{2+} transients are significantly smaller in myocytes isolated from female mice when compared to males (Farrell et al., 2010).

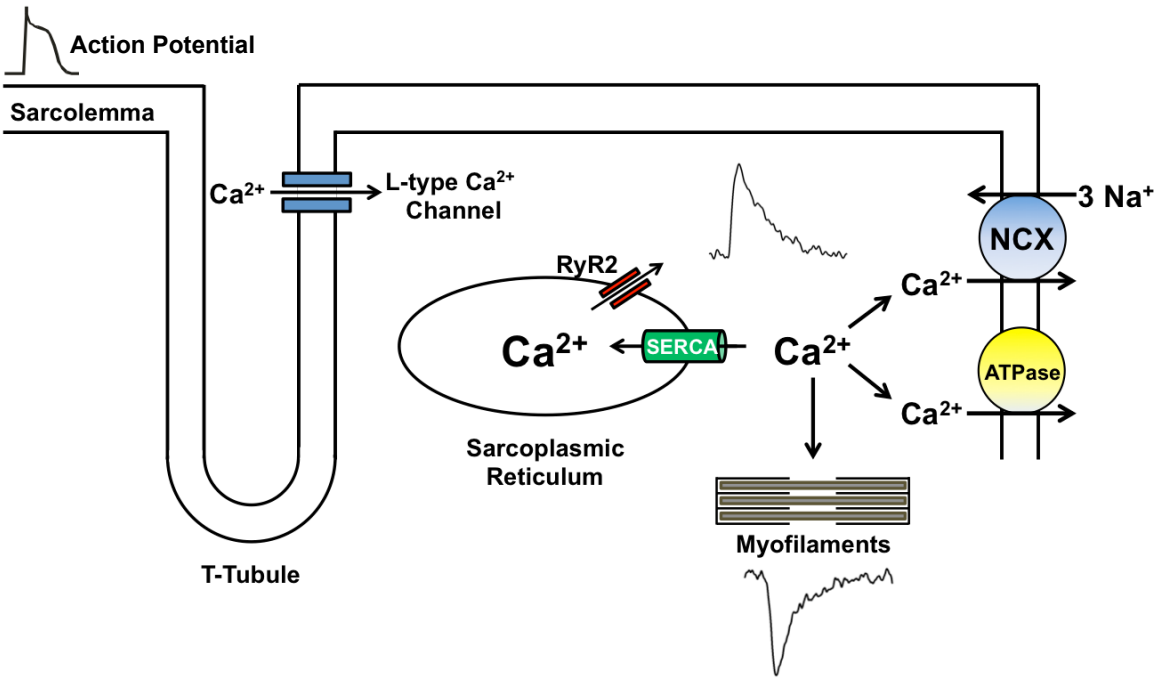


Figure 1.2. Schematic of the excitation-contraction coupling pathway in a ventricular myocyte. Membrane depolarization triggered by the action potential promotes Ca^{2+} influx through the voltage-dependent, L-type Ca^{2+} channel. The incoming Ca^{2+} then goes onto to interact with ryanodine receptors (RyR2) to trigger Ca^{2+}-induced-Ca^{2+}-release (CICR) from the sarcoplasmic reticulum (SR). This gives rise to the Ca^{2+} transient. The increased $[Ca^{2+}]_i$ interacts with the myofilaments to induce a contraction. Following contraction, Ca^{2+} is taken back up into the SR via the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is extruded from the cell via the Na^+/Ca^{2+} exchanger (NCX).

Additionally, the resulting contraction is smaller in myocytes from females compared to cells isolated from males (Farrell et al., 2010). As previously described, a number of components of the EC coupling pathway work together to initiate the Ca^{2+} transient and the resulting contraction. Therefore, this suggests that estrogen may influence various components of the EC coupling pathway and alter myocyte contractile function through changes in Ca^{2+} movement within the cardiomyocyte.

iii. β-adrenergic Signalling in the Myocardium

Various components of the EC coupling pathway are modulated by sympathetic stimulation. Activation of the β_1-adrenergic receptor (β_1-AR) acts as a positive inotrope to increase cardiac contractile force (Bers, 2002). Additionally, β_1-AR stimulation has positive lusitropic effects, as it speeds relaxation of the myocardium through rapid reduction in $[Ca^{2+}]_i$ (Li et al., 2000b). When catecholamines bind to the β_1-AR, the heterotrimeric G-protein, G_s, is activated (Bers, 2002) (Fig. 1.3). The α-subunit of G_s dissociates from the $\beta\gamma$-subunit and goes on to stimulate adenylyl cyclase (AC). AC stimulates the conversion of ATP into cyclic AMP (cAMP), which in turn activates protein kinase A (PKA). PKA phosphorylates numerous components of the EC coupling pathway including the L-type Ca^{2+} channel, RyR, PLB, as well as troponin I on the myofilaments (Bers, 2002).

Phosphorylation of PLB by PKA elicits the majority of the positive lusitropic effect observed with β_1-AR activation (Li et al., 2000b). β_1-AR stimulation speeds the re-uptake of Ca^{2+} into the SR by causing PLB to dissociate from SERCA. As well, phosphorylation of troponin I reduces myofilament Ca^{2+} sensitivity and causes a

more rapid dissociation of Ca^{2+} from the myofilaments, which also functions to speed relaxation (Kentish et al., 2001). The increased speed of Ca^{2+} re-uptake into the SR leads to increased SR Ca^{2+} stores. Together, the larger SR Ca^{2+} stores and increased $I_{Ca,L}$ mediate the positive inotropic effects observed following application of a β_1-AR agonist. Phosphorylation of RyR by PKA also causes an increase in RyR channel open probability (Valdivia et al., 1995). It has also been reported that RyR phosphorylation may increase Ca^{2+} release from the SR (Viatchenko-Karpinski and Gyorke, 2001), although, this remains controversial (Song et al., 2001).

As mentioned previously, Ca^{2+} overload can be detrimental to the heart. It is well established that β_1-AR stimulation can cause Ca^{2+} loading in myocytes (Sipido, 2006). It is thought that this alteration in Ca^{2+} movement within the cardiomyocyte is due to an increase in $I_{Ca,L}$ and enhanced SERCA activity (Volders et al., 2000). In fact, SR Ca^{2+} loading can become so excessive that spontaneous SR Ca^{2+} release can occur (Volders et al., 2000). This can lead to the generation of early and delayed after depolarizations which can result in various cardiac arrhythmias (Volders et al., 2000). Together, these observations help explain the wide-spread use of β-blockers in patients suffering from heart failure in order to suppress Ca^{2+} loading in the heart.

A limited number of studies have investigated the effects of estrogen in the presence of sympathetic stimulation. Cross et al. (2002) used Langendorff hearts to demonstrate that females are protected from ischemia-reperfusion injury in the presence of the β_1-AR agonist, isoproterenol. They concluded that the protection observed in the females was mediated by estrogen-induced, nitric oxide generation (Cross et al., 2002). Taken together, these results suggest that estrogen may be

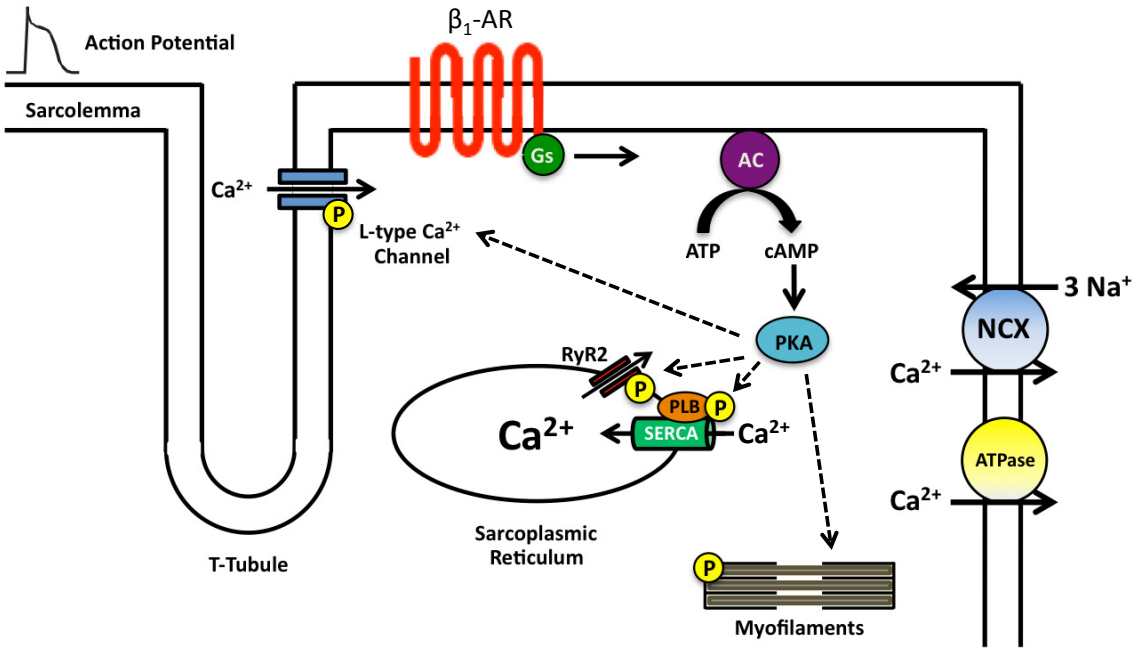


Figure 1.3. Schematic of β_1-adrenergic receptor stimulation in a ventricular myocyte. Stimulation of the β_1-AR activates the G protein, G_s. G_s goes on to activate adenylyl cyclase (AC). AC stimulates the conversion of ATP into cyclic AMP (cAMP), which in turn activates protein kinase A (PKA). PKA phosphorylates numerous components of the EC coupling pathway including the L-type Ca^{2+} channel, RyR, PLB, and troponin I, which is located on the myofilaments.

cardioprotective in situations of high-stress which initiate sympathetic activation.

III. Estrogen Signalling in the Myocardium

I. Overview of Estrogen

Estrogens are sex steroid hormones that are derived from cholesterol (Czubryt et al., 2006). There are three forms of estrogen: estrone, estradiol, and estriol (Burger, 2001). However, the most potent estrogen in the human body is 17β-estradiol (Kalus, 2008). Estrogen has a variety of physiological roles which include formation of secondary sex characteristics, maintenance of bone mass, production of adipose tissue, and acceleration of metabolism (Cagnacci et al., 1992). The enzyme aromatase is responsible for the conversion of androgens to estrogens (Simpson et al., 1994). The majority of estrogen synthesis in pre-menopausal women occurs in the ovaries, where aromatase expression is the highest. However, in post-menopausal women, the principal aromatase expression is shifted to adipose tissue and skin fibroblasts (Simpson et al., 1994). Thus, in pre-menopausal women, it is the fluctuating levels of estrogen secreted from the ovaries that are thought to be responsible for cardioprotection. This cardioprotection is lost when adipose tissue and skin fibroblasts become the main source of more constant low levels of estrogen, which are seen in post-menopausal women.

Stumpf et al. were the first to discover that cardiomyocytes contained estrogen receptors (Stumpf et al., 1977). They used tritium-labeled estradiol and found that various areas of the heart contained estrogen receptors, including the left and right ventricles and the left and right atria. Additionally, cardiac fibroblasts

contain functional estrogen receptors (Grohe et al., 1997). While the effects of estrogen signalling in cardiac fibroblasts is poorly understood, a study has shown that estrogen may play a role in fibroblast proliferation (Lee and Eghbali-Webb, 1998). It is now known that estrogen is capable activating downstream signalling cascades by binding to estrogen receptors in the nucleus and on the plasma membrane (Revankar et al., 2005). The effects of estrogen are mediated via genomic and non-genomic signalling pathways, as described in detail below (Bjornstrom and Sjoberg, 2005).

ii. Genomic Signalling Pathway: Estrogen Receptors α and β

Estradiol mediates its genomic effects on the cardiovascular system by binding to the two nuclear estrogen receptors, known as estrogen receptor α (ERα) and estrogen receptor β (ERβ) (Grohe et al., 1997). In cardiomyocytes, expression levels of ERβ are similar between males and females (Grohe et al., 1998). However ERα expression levels are higher in pre-menopausal women when compared to age-matched men (Ihionkhan et al., 2002). When estradiol binds to either of these receptors, the complex translocates to the nucleus and is capable of altering the transcription of certain genes. The genomic changes elicited by estradiol are mediated by the nuclear ERs in three ways: 1. ER binds directly to DNA 2. ER binds indirectly to DNA, or 3. Ligand-independent binding (Hall et al., 2001). These mechanisms for genomic estrogen signalling have been identified in a variety of tissues, however, it is not yet known which of these binding profiles are specifically

responsible for genomic estrogen signalling in the myocardium. Therefore, a brief overview of each mechanism is provided below.

ERs are capable of directly binding to DNA to modify gene transcription (Hall et al., 2001). Upon estradiol binding to the activator function-2 domain of either ERα or ERβ, the receptor dimerizes. The dimer then goes on to bind to the estrogen response elements located on the DNA, which leads to the recruitment of co-activators or co-repressors (Rosenfeld and Glass, 2001). This results in enhanced or reduced gene expression. For example, it is likely that estrogen down-regulates transcription of the L-type Ca^{2+} channel, as there is a dramatic increase in L-type Ca^{2+} channel expression in ER knock-out mice (Johnson et al., 1997).

Transcription can also be altered by estradiol in genes that do not contain an estrogen response element (Bjornstrom and Sjoberg, 2005). Approximately 33% of genes that are influenced by estradiol binding to ER do not have estrogen response elements (O'Lone et al., 2004). In this case, following binding of estradiol to the ER, the ER goes on to bind to an alternative transcription factor. This mechanism is frequently referred to as transcriptional cross talk (Gottlicher et al., 1998). ERs are also capable of signalling in the absence of estrogen binding (Curtis et al., 1996). However, the mechanism behind this signalling is poorly understood. Taken together, these studies suggest 3 potential signalling pathways through which activated ERs are capable of inducing genomic changes following estrogen binding. It is clear that further investigations are required to determine if any or all of these pathways are responsible for the genomic changes observed with estrogen binding to the myocardium.

iii. Non-genomic Signalling Pathway: Estrogen Receptors α and β and GPER

Recent studies suggest that, aside from its established genomic effects, estrogen is also capable of mediating non-genomic actions (Simoncini and Genazzani, 2003). Non-genomic actions occur rapidly and are often coupled to protein-kinase activation. It is thought that estrogen may mediate rapid signalling via estrogen receptors that are bound to the plasma membrane, which go on to activate both G_{S_α} and G_{q_α} (Kuroki et al., 2000). For example, 17β-estradiol has been shown to rapidly mobilize intracellular Ca^{2+} (Improta-Brears et al., 1999), stimulate adenylate cyclase activity (Aronica et al., 1994), and increase cyclic AMP production in cultured breast cancer and uterine cells (Aronica et al., 1994). Activation of G proteins can lead to activation of the extracellular signal-regulated kinase (ERK), cAMP, and phosphatidylinositol 3-kinase (PI3K) signalling pathways (Razandi et al., 2004). Although relatively little is known about the non-genomic effects of estrogen, they are thought to contribute to rapid cardioprotection during ischemic cardiac injury and myocardial remodelling (McHugh et al., 1995; Node et al., 1997).

The non-genomic effects of estrogen have been demonstrated in isolated guinea-pig cardiomyocytes (Jiang et al., 1992). Acute application of a supra-physiological concentration of 17β-estradiol rapidly reduces myocyte contraction (Jiang et al., 1992). However, whether this is caused by a change in intracellular Ca^{2+} is not known because intracellular Ca^{2+} levels were not quantified in this study. As previously mentioned, it is thought that the beneficial effects of estrogen on the heart are due to changes in Ca^{2+} movement throughout the cell. Therefore, further

studies are required to understand the effects of acute application of estrogen on isolated ventricular myocytes.

Recently, a novel G protein-coupled receptor (GPCR) has been identified, known as G protein-coupled estrogen receptor (GPER) (Filardo, 2002). It has been suggested that GPER may be responsible for estrogen-mediated, non-genomic signalling. GPCRs are proteins that contain 7 trans-membrane domains (Ji et al., 1998). Ligand binding to GPCRs activates heterotrimeric G proteins which initiate various downstream signalling cascades. Estrogen was first shown to bind to GPER in a line of breast cancer cells which do not express either of the nuclear ERs (Filardo, 2002). In this cell line, application of 17β-estradiol rapidly activates the mitogen-activated protein kinases ERK-1 and ERK-2 (Filardo, 2002). These findings suggest that estrogen may have acute effects on other cells that express GPER, however, relatively little is know about this pathway.

Following identification of GPER, a novel GPER agonist known as G-1 was discovered (Bologa et al., 2006). G-1 was shown to bind to GPER with high affinity and showed no binding affinity for either ERα or ERβ (Bologa et al., 2006). To date, most studies on the role of GPER in the heart have examined the effect of G-1 on ischemia-reperfusion injury. Deschamps and Murphy (2009) demonstrated that GPER is present on the myocardium and that similar protein levels are seen in male and female rats. As well, they demonstrated that activation of GPER by G-1 is cardioprotective in Langendorff hearts subjected to ischemia-reperfusion injury (Deschamps and Murphy, 2009). They attributed the protective effects of G-1 to activation of the PI3K pathway. Together, these results demonstrate that that G-1 is

capable of signalling via GPER in the intact whole heart. However, whether G-1 able to modify cardiomyocyte contractions and Ca^{2+} homeostasis has yet to be determined.

IV. The Human Menstrual Cycle and Mouse the Estrous Cycle

i. The Human Menstrual Cycle

Estrogen levels are not constant in pre-menopausal women. Rather, they fluctuate during the human menstrual cycle. The human menstrual cycle is a reproductive process in females that begins at approximately 12 years of age (Lentz, 2012). The menstrual cycle has a mean duration of 28 days and is divided into two phases, known as the follicular phase and the luteal phase, which are separated by ovulation. In young, sexually-mature females, both the follicular and luteal phases last approximately 14 days (Lentz, 2012). The follicular phase begins with a rise in follicle stimulating hormone (FSH) levels from the anterior pituitary which promotes follicle growth resulting in a single mature follicle. As the follicles grow, they secrete increasing levels of 17β-estradiol to promote endometrial development. When the follicle has almost matured, estradiol levels reach a threshold in which a positive feedback loop is initiated that results in ovulation (Lentz, 2012). Thus, high plasma estradiol levels are detected by the hypothalamus which provides feedback to the ovaries to cease production of 17β-estradiol. During ovulation, 17β-estradiol levels fall and there is a surge in luteinizing hormone (LH) levels from the anterior pituitary. After the ovum has left the ovary, the corpus luteum left behind secrets large amounts of progesterone. If fertilization does not

occur, levels of progesterone decline and menstruation occurs. Therefore, the human menstrual cycle is characterized by cyclical changes in a number of different hormones, including estrogen (Lentz, 2012).

ii. The Mouse Estrous Cycle

Analogous to the human menstrual cycle, rodents experience an estrous cycle which is characterized by fluctuating levels of sex hormones (Butcher et al., 1974). The estrous cycle has an approximate duration of 4-6 days in laboratory mice, and does not begin until mice reach sexual maturity at 42-56 days, which coincides with vaginal opening (Nelson et al., 1982). The estrous cycle can be tracked in mice through observation of vaginal cytology which in turn, correlates with hormonal fluctuations.

Four primary estrous stages have been identified: diestrus, proestrus, estrus, and metestrus (Barkley and Bradford, 1981). Proestrus corresponds to the follicular phase observed in humans, whereas, estrus, metestrus, and diestrus occur during the luteal phase. These stages can be distinguished based on vaginal cytology, where a sample of vaginal cells are placed on a slide, fixed, and subsequently stained (Nelson et al., 1982). Nelson et al. have shown that vaginal smears from diestrus consist primarily of leukocytes as well as a small number of nucleated and cornified cells (Nelson et al., 1982). Vaginal smears from proestrus contain mainly nucleated cells and few of the other cell types. Estrus is characterized by a thick vaginal smear that contains only cornified cells. Finally,

metestrus vaginal smears contain cornified cells as well as leukocytes and some nucleated cells.

While the four estrous stages are well defined, few studies have sought to quantify corresponding hormone levels in the mouse model, therefore only ranges of estrogen concentrations can be assumed (Table 1.1) (Bergman et al., 1992; Campbell et al., 1976; Saito et al., 2009; Walmer et al., 1992). Proestrus has been characterized as the estrous stage with the highest estradiol levels, while the majority of studies report that estradiol levels are lowest in estrus.

Interestingly, recurrent, regular estrous cycling in laboratory mice housed in conventional animal care facilities occurs infrequently (Barkley and Bradford, 1981). Female pheromones have been shown to cause a suppression of estrous cycling and prolongation of the cycle in female mice housed in groups (Whitten, 1959). However, exposing these group-housed females to male pheromones can induce regular estrous cycling and may also synchronize the females' cycles; this phenomenon is referred to as the "Whitten Effect" (Whitten, 1966). Therefore, group-housed females in typical animal care facilities may not exhibit regular estrous cycles.

V. Estrogen Targets in the EC Coupling Pathway

i. Estrogen and the EC Coupling Pathway

The ovariectomized (OVX) mouse has been an invaluable tool in understanding the effects of chronic ovarian estrogen deprivation. This model has been used to investigate the cardiovascular effects of ovary removal. To date, the

Table 1.1. Mouse plasma/serum 17β-estradiol levels (pg/ml) from literature.

Source	P/S	Method	D	P	E	M
Campbell, 1976	S	RIA	-	~24	-	~4
Saito, 2009	P	-	60 ± 10	60 ± 9	17.1 ± 3	19.6 ± 2
Walmer, 1992	S	FIA	~23	~56	~22	~34

Abbreviations: P – plasma sample, S – serum sample, RIA – radioimmunoassay, FIA – fluorescence immunoassay, D – diestrus, P – proestrus, E – estrus, M – metestrus.

results of these studies provide evidence that several components of the EC coupling pathway are likely affected by the chronic actions of estrogen. Fractional shortening and Ca^{2+} transient amplitudes are significantly larger in myocytes isolated from OVX rats when compared to those isolated from sham-operated controls (Curl et al., 2003). Interestingly, supplementation of OVX animals with 17β-estradiol returns fractional shortening and Ca^{2+} transient amplitudes to levels that are comparable to sham-operated controls (Curl et al., 2003). Along with the observed increase in intracellular Ca^{2+}, OVX myocytes also have reduced myofilament Ca^{2+} sensitivity (Schaible et al., 1984). This is thought to be due to a decrease in myosin ATPase activity (Schaible et al., 1984). Taken together, these results suggest that estrogen may modulate various components within the EC coupling pathway. Intracellular Ca^{2+} levels are increased in myocytes isolated from OVX mice, therefore, it is possible that estrogen may function to decrease $I_{Ca,L}$, RyR activation, and SR Ca^{2+} stores, as each of these components are known to influence Ca^{2+} homeostasis. Additionally, estrogen may also sensitize the myofilaments to Ca^{2+} through an increase in myosin ATPase activity. However, further studies are required, as relatively little is known about the chronic effects of estrogen on myocyte contractile function.

The non-genomic effects of estrogen on components of the EC coupling pathway can be studied by directly applying 17β-estradiol on isolated cardiomyocytes. Effects that occur rapidly following application of 17β-estradiol would be mediated by non-genomic signalling. Acute application of 17β-estradiol has been shown to rapidly inhibit $I_{Ca,L}$ in isolated rat ventricular myocytes (Berger et

al., 1997; Nakajima et al., 1999). Additionally, a negative inotropic effect is observed upon application of 17β-estradiol in isolated human myocardium (Sitzler et al., 1996). Thus, acute application of 17β-estradiol is associated with decreased $I_{Ca,L}$ as well as a reduction in fractional shortening. Therefore, it is likely that the acute effects of estradiol are associated with a decrease in intracellular Ca^{2+}, as a smaller $I_{Ca,L}$ would be expected to decrease peak Ca^{2+} transients. Taken together, these results suggest that both acute and chronic exposure to estrogen function to suppress myocyte contractile function through reduced intracellular Ca^{2+} release. As high levels of intracellular Ca^{2+} have been associated with various cardiovascular diseases (Katz and Reuter, 1979), it is possible that susceptibility to adverse cardiovascular events may vary with cyclical changes in estrogen levels. However, little is known about the effect of the estrous cycle on Ca^{2+} homeostasis in the heart.

Although information is limited, one study did examine the effects of cyclical estrogen levels characteristic of the rodent estrous cycle on cardiac function. This study investigated the effect of the estrous cycle on action potential duration (APD) and ventricular repolarization (Saito et al., 2009). Action potential duration at 50% repolarization (APD50) and APD90 were found to be significantly longer in estrus due to a decrease in the transient outward K^+ current ($I_{to,f}$) as well as decreased ultra-rapid delayed rectifier K^+ current ($I_{K,slow}$) (Saito et al., 2009). Both of these currents play integral roles in ventricular repolarization (Brouillette et al., 2004; Xu et al., 1999). These results indicate that cyclical changes in estrogen can modify action potential configuration in the heart.

ii. Estrogen and β-adrenergic Signalling in the Myocardium

Various studies suggest that estrogen can alter cardiovascular function through actions on the β-AR. An up-regulation of β_1-AR has been observed in the hearts of OVX rats (Thawornkaiwong et al., 2003). However, following estrogen replacement in OVX rats, β_1-AR expression is restored to levels similar to the sham-operated controls (Kam et al., 2004). Acute application of estradiol can reduce both heart rate and developed pressure in a Langendorff working heart in the presence of ISO (Li et al., 2000a). As well, estradiol reduces $I_{Ca,L}$ in ventricular myocytes treated with ISO (Li et al., 2000a). Taken together, these results suggest that chronic exposure to 17β-estradiol down-regulates β_1-AR in individual cardiomyocytes. Additionally, the acute studies suggest that 17β-estradiol may attenuate the effects of sympathetic stimulation in the whole heart and in individual cardiomyocytes.

VI. Hypotheses

1. Contractile function and Ca^{2+} concentrations fluctuate in cardiomyocytes isolated from the hearts of mice in the four stages of the estrous cycle.
2. Myocytes isolated from mice in estrus, when estradiol levels are lowest, are the most responsive to sympathetic stimulation by the β-adrenergic agonist, isoproterenol.
3. Acute application of 17β-estradiol or G-1 modifies contractions and Ca^{2+} transients in isolated mouse ventricular myocytes.

VII. Objectives

The objectives of the present study were to 1) determine whether group-housed females display the typical cyclical changes associated with the estrous cycle; 2) determine if contractile function and Ca^{2+} concentrations fluctuate in myocytes isolated from mice in each of the four stages of the estrous cycle; 3) determine if myofilament Ca^{2+} sensitivity fluctuates with the estrous cycle; 4) determine if the effects of the estrous cycle on myocyte contractile function and Ca^{2+} concentrations persist at physiological pacing rates; 5) determine if responsiveness to catecholamines fluctuates with the estrous cycle; 6) determine if acute application of 17β-estradiol or G-1 modifies contractions and Ca^{2+} concentrations in myocytes isolated during the four stages of the estrous cycle.

Chapter 2: Methods

I. Animals

Experiments were performed in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (CCAC, Ottawa, ON: Vol 2, 1984). The Dalhousie University Committee on Laboratory Animals approved all experimental protocols. Most experiments used female C57BL/6 mice (young adult: ~3 mos) obtained from Charles River Laboratories (St. Constant, QC). However, in one set of experiments aged C57BL/6 female mice (~24 mos) were also used. Animals were housed in the Carleton Animal Care Facility at Dalhousie University on a 12-hour light/dark cycle with food and water ad libitum. Animals were housed in groups of five mice per cage. The cages were covered with micro-isolator lids.

II. Vaginal Smears

Female mice were exposed to bedding collected from the cages of young adult male (~3 mos) mice in order to induce cycling. This bedding was placed in the females' cages daily for the seven days prior to initial vaginal smear collection, as described previously (Campbell et al., 1976). Vaginal smears were collected daily between 9:00am and 11:00am. Tooth picks and loose cotton were used to create small swabs to obtain vaginal smears. Swabs were dipped in sterile saline before use. Each mouse was held by the base of the tail while the swab was gently inserted into the vaginal opening and rotated to recover cells from the vaginal wall. Cells were then transferred to a slide, allowed to dry, and fixed using 100% methanol.

Slides were subsequently stained with eosin and thiazine (Dip Quick stain Set; Jorvet, Loveland, CO). Stained slides were fixed using a toluene solution (Permount; Fisher Scientific, Ottawa, ON) on a cover slip.

Two groups of female mice were compared to confirm that male pheromones were capable of inducing female cycling. One group of females was exposed to the bedding collected from a cage housing male mice and the other was not. These animals were housed in the same room within the animal care facility. Vaginal smears were collected for 14 consecutive days from mice in each group. It was confirmed that exposure to male pheromones rapidly induced regular cycling, as described in detail in the results section. All subsequent experiments were conducted on females that were exposed to male pheromone-containing bedding for 7 consecutive days followed by exposure twice/week to maintain cycling.

III. Classification of Vaginal Cytology

Vaginal smears were stained as described above and viewed under an inverted microscope (Olympus IMT-2; Carsen Group Inc., Markham, ON) at 100X magnification. The estrus stage was categorized for each animal based upon the cell types present. Four estrous stages were distinguished: diestrus (D), proestrus (P), estrus (E), and metestrus (M) (Fig. 2.1). Diestrus smears were classified by the presence of leukocytes and some nucleated and cornified cells (Fig. 2.1A). Proestrus was classified by the presence of nucleated cells with or without leukocytes (Fig. 2.1B). A smear was categorized as estrus if only cornified cells were present (Fig.

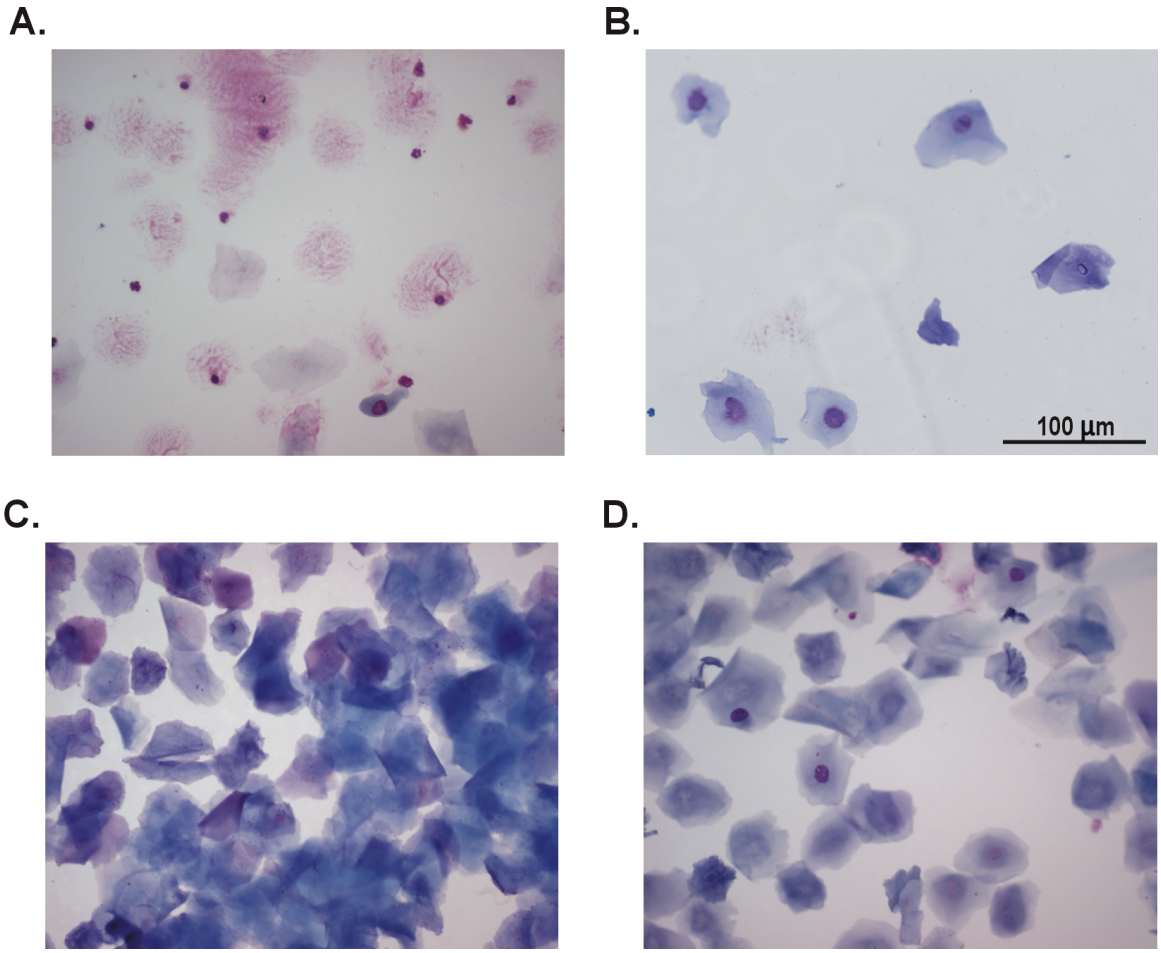


Figure 2.1. Estrous stages defined by vaginal cytology. Vaginal smears were collected from sexually mature C57BL/6 mice throughout the estrous cycle. Slides were stained using eosin and thiazine. **A:** Diestrus smears predominantly contained leukocytes and a small number of nucleated and cornified cells. **B:** Proestrus smears were characterized by the presence of nucleated cells and a few leukocytes. **C:** Estrus smears were classified by the sole presence of darkly stained cornified cells. **D:** Metestrus smears contained lightly stained cornified cells and a small number of leukocytes and nucleated cells.

Table 2.1. Vaginal cytology present throughout the estrous cycle.

Estrous Stage	Cell Type		
	Leukocyte	Cornified	Nucleated
Diestrus	xxx	X	x
Proestrus	x		xxxx
Estrus		xxxxx	
Metestrus	x	xxx	x

Approximate quantity (%) of each cell type present in vaginal smears collected during diestrus, proestrus, estrus, and metestrus. X represents approximately 20%.

2.1C). Metestrus smears contained both lightly stained cornified cells and a small number of leukocytes and nucleated cells (Fig. 2.1D). The cytology of these stages are summarized in Table 2.1. Mice often exhibited intermediate stages (ie. proestrus/estrus), however these animals were not used for experiments. All experiments conducted within this thesis were performed using mice displaying one of the four main stages of the estrous cycle, unless otherwise indicated.

IV. Ventricular Myocyte Isolation

Following determination of the estrous stage, the mouse was transferred to a clean cage within the Animal Care Facility and transported to the lab. The mouse was given food and water and placed in a quiet area within the lab for 30 mins. Sodium pentobarbital (IP, 200mg/kg; CDMV, Saint-Hyacinthe, QC) and heparin (IP, 3000U/kg; Pharmaceutical Partners of Canada, Richmond, ON) were co-injected to anesthetise the mouse and prevent blood clotting, respectively, during the isolation procedure. The mouse was weighed following the loss of consciousness. Anaesthesia was confirmed by the absence of the pedal withdrawal reflex. The animal was placed on the surgical table and the ribs were exposed using a sternal incision. The pericardium was removed to expose the heart. The aorta was severed before removing the heart from the chest cavity. The aorta was cannulated *ex-vivo*, secured with a suture, and the heart was then perfused (Fig. 2.2) retrogradely at 2.2 ml/min with oxygenated nominally Ca^{2+}-free buffer at 37˚C . The Ca^{2+}-free buffer was composed of the following (in mM): 105 NaCl, 5 KCl, 1 $MgCl_2$, 0.33 NaH_2P0_4, 25

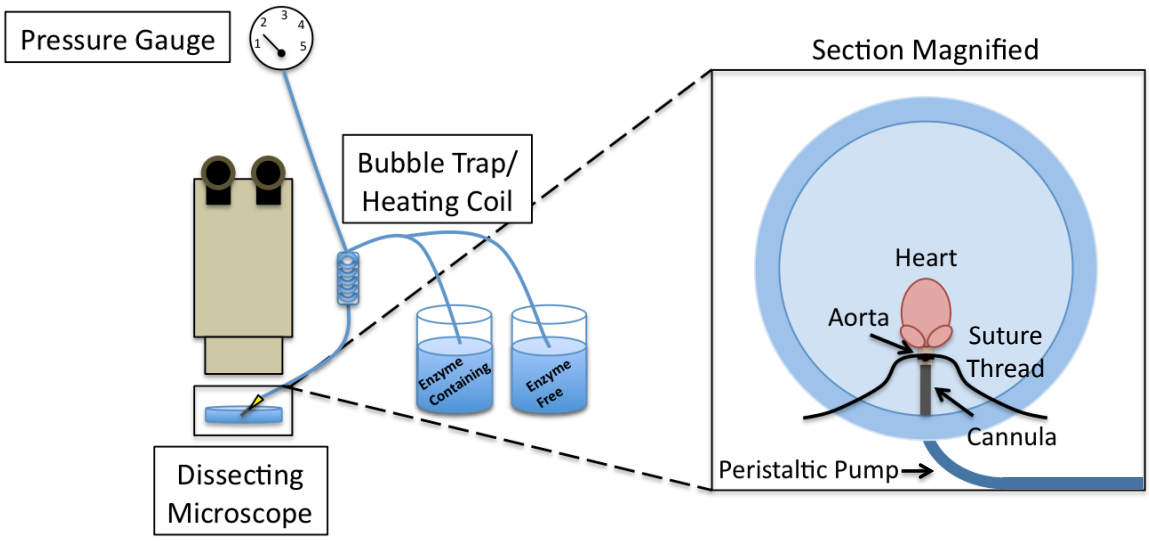


Figure 2.2. Schematic of ventricular myocyte isolation set-up. Isolation procedure is explained in detail within the methods section.

HEPES, 20 glucose, 3 Na-pyruvate, 1 lactic acid (pH 7.4 with NaOH). After 10 mins of perfusion, the solution was switched to the above Ca^{2+}-free buffer supplemented with collagenase type II (8.0 mg/30ml; Worthington, Lakewood, NJ), dispase II (3.4 mg/30ml; Roche Diagnostics, Laval, QC), trypsin (0.5 mg/30ml; Sigma Aldrich, Oakville, ON), and 50 μM $CaCl_2$. The heart was perfused with the enzyme-containing buffer for approximately 8 mins. The isolation buffers were bubbled with 100% O_2 (Praxair, Halifax, NS) and warmed to 37˚C using a heating coil (Radnoti Glass Technology Inc. Monrovia, CA) heated by a circulating water bath (Haake; Scientific, Ottawa, ON). A peristaltic pump (Piper model P; Fred A. Dungey Inc., Agincourt, ON) was used to pump the solution through the heart, and any air bubbles were removed by a bubble trap located within the heating coil. Following enzymatic digestion, the ventricles were separated from the atria and cut into small pieces in a high-potassium buffer containing (in mM): 45 KCl, 3 $MgSO_4$, 30 KH_2P0_4, 50 L-glutamic acid, 20 taurine, 0.5 EGTA, 10 HEPES, 10 glucose (pH to 7.4 with KOH). The tissue was rinsed with this buffer three times to ensure removal of the enzyme-containing buffer. The ventricular tissue was swirled in buffer to dissociate myocytes, and the cell suspension was filtered through a 225μm polyethylene mesh to prevent the transfer of any large pieces of tissue.

V. Field Stimulation: Intracellular Ca^{2+} and Contraction Measurements

The myocytes were incubated in the dark with fura-2 AM (5 μM) for 20 minutes in a plexiglass chamber with a glass bottom secured to the stage of an inverted microscope (Nikon Eclipse TE200; Nikon Canada, Mississauga, ON). The

microscope was placed within a Faraday cage to minimize electrical noise. The Faraday cage was covered with black vinyl to prevent surrounding light from interfering with the fluorescence recordings. As well, the microscope was placed on a custom-made air table, so that any surrounding vibrations would not affect the experiment. Myocytes were visualized using an oil immersion 40x lens (Nikon S-Fluor, numerical aperture 1.30, Nikon Canada Inc.). Following incubation for 20 mins, cells were superfused with HEPES buffer containing (in mM): 135.5 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, 1.0 $CaCl_2$ (pH to 7.4 with NaOH). Superfusion was controlled by a peristaltic pump (Minipulse 3, Gilson Inc.; Middleton, WI) at a flow rate of ~3 ml/min. The buffer was warmed to 37˚C using a circulating water bath (Polystat, Model # 12112-10, Cole Parmer, Vernon Hills, IL).

Figure 2.3 provides an illustration of the experimental set-up for field stimulation. Fluorescence and cell shortening were measured simultaneously by dividing the microscope light between a video camera (Philips, Markham, Ontario) and a photomultiplier tube (Photon Technologies International (PTI), Birmingham, NJ) using a dichroic cube (Chroma Technology Corp., Rockingham, VT). Whole-myocyte fluorescence was obtained by closing the aperture of the photomultiplier tube to fit the precise size of the cell observable through the viewing window. A DeltaRam fluorescence system (PTI) was used to alternately excite myocytes with 340 nm and 380 nm light. Fluorescence emitted at 510 nm was recorded for both 340 nm and 380 nm wavelengths at a rate of 200 samples/second with Felix software (PTI). Recordings were made for a period of 10 secs at 5 – 8 minute intervals depending upon the experiment. Following the experiment, background

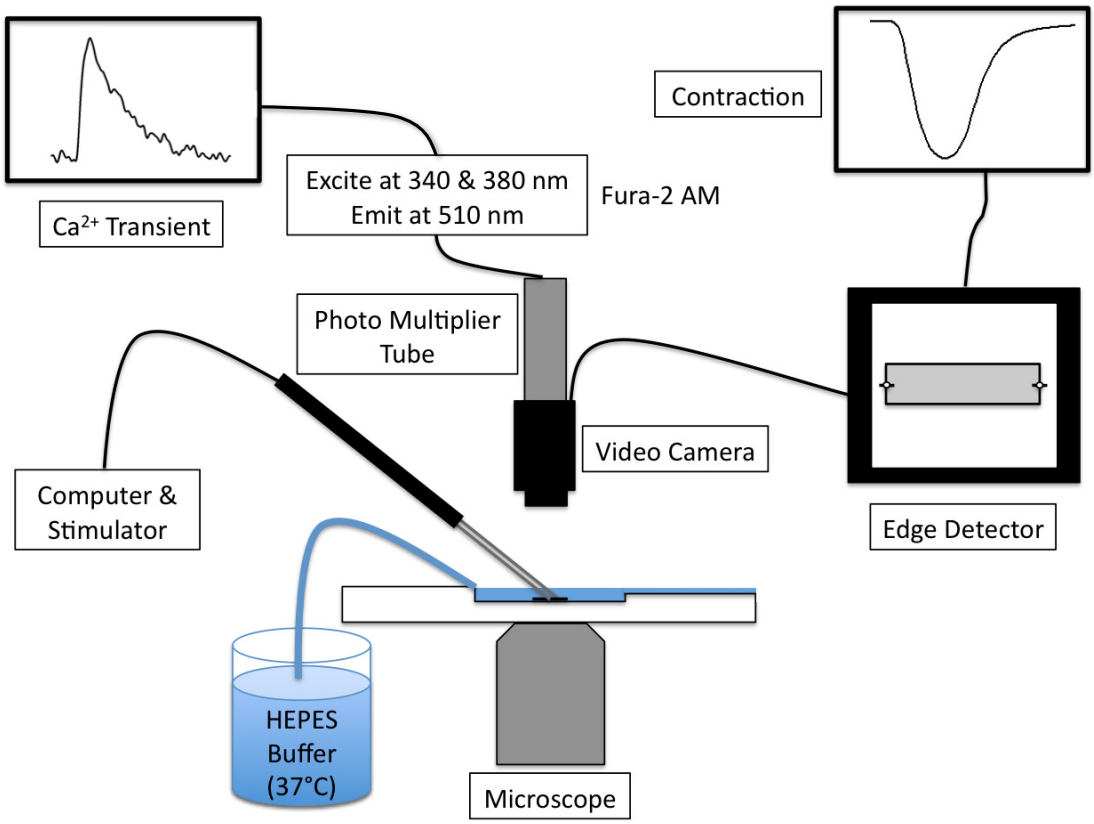


Figure 2.3. Schematic of field stimulation set-up. Each component is described in detail within the methods section.

fluorescence was recorded for each cell. Background fluorescence was recorded from an area close to the cell that contained no myocytes or cell fragments.

Cell length was measured by a video edge detector at a rate of 120 samples/second (Model # 105; Crescent Electronics, Sandy, UT). Two platinum electrodes were placed on either side of the microscope field of view within the plexiglass chamber. Bipolar pulses (3 ms, 30 – 70 mA) were generated by a stimulus isolation unit (Model # SIU- 102; Warner Instruments, Hamden, CT) and the rate of myocyte stimulation was controlled by pClamp 8.2 software (Molecular Devices, Sunnyvale, CA). Myocytes were stimulated at 4 and/or 6 Hz depending upon the experiment. For experiments where drugs were applied, fluorescence and cell shortening were recorded following myocyte exposure to control, vehicle-containing, and drug-containing solutions.

VI. Drug Exposure

Cells were exposed to the non-selective β-adrenergic agonist, isoproterenol, to increase intracellular Ca^{2+} levels. Serial dilutions from 1 nM to 1 μM were administered at 5-minute intervals. No vehicle control was needed as isoproterenol was dissolved in water. In other experiments, cardiomyocytes were acutely exposed to 17β-estradiol. Serial dilutions from 0.1 nM to 1 μM were administered at 7-minute intervals. All solutions contained the maximum concentration of DMSO (0.001%) to control for solvent effects. In another series of experiments, ventricular myocytes were acutely exposed to the GPER agonist, G-1. Serial dilutions from 0.1 nM to 100 nM were added at 7-minute intervals. All solutions contained the

maximum concentration of DMSO (0.001%). A previous experiment confirmed that 0.01% DMSO had no effect on contraction size or Ca^{2+} transients. Even so, time controls were measured in order to ensure that basal cardiac contractile function did not change over the time frame of both the 17β-estradiol and G-1 experiments.

VII. Heart Isolation and Myofilament Ca^{2+} Sensitivity

Mice in each of the four stages of the estrous cycle were anaesthetized and the thoracic cavity was exposed using the protocol for myocyte isolation described above. A small cut was made in the right atrium, and the heart was perfused with 30 ml sterile saline injected into the left ventricle through a 25-gauge needle (BD, Franklin Lakes, NJ). Hearts were then excised from the chest and the atria were removed. The ventricles were transversely sectioned in half and flash-frozen using liquid nitrogen. Ventricular samples were stored at -80°C until they were assessed for myofilament Ca^{2+} sensitivity.

The following protocols for myofilament isolation and the subsequent actomyosin MgATPase assay were carried out by Dr. W. Glen Pyle at the University of Guelph in Guelph, Ontario. Ventricular sections were homogenized in ice-cold standard buffer. The standard buffer was composed of the following (in mM): 60 KCl, 30 imidazole (pH 7.0), 2 $MgCl_2$, 0.01 leupeptin, 0.1 PMSF, 0.2 benzamidine, and 0.1 cantharidin. The ventricular homogenate was centrifuged at 14,100 g for 15 mins at 4°C. The resulting pellet was resuspended for 45 mins in ice cold Standard Buffer supplemented with 1% Triton X-100. This solution was centrifuged at 1,100 g for 15 mins at 4°C. The final resulting pellet was washed three times in the above

ice cold Standard Buffer. The purified myofilaments were either stored on ice for a maximum of 2 h or frozen for gel electrophoresis.

Following myofilament purification, a spectrophotometric actomyosin MgATPase assay was used to assess myofilament function. Purified myofilaments (25 mg) were incubated in activating solutions containing various levels of free Ca^{2+}. Ca^{2+} levels were (in mM): 0.001, 0.11, 0.42, 0.54, 0.71, 0.95, 10.8, or 124. These varying levels of Ca^{2+} were established using combinations of activating and relaxing buffers. Free Ca^{2+} was calculated using the Max-Chelator program Winmaxc (version 2.05). The purified myofilaments were incubated for 10 mins in activating buffers at 32°C. This reaction was attenuated with 10% trichloroacetic acid. The production of inorganic phosphate was measured by adding equal volumes of 0.5% $FeSO_4$ and 0.5% ammonium molybdate in 0.5 M H_2SO_4. The absorbance was read at 630 nm.

VIII. Chemicals

Fura-2 AM was obtained from Invitrogen (Burlington, ON). A stock solution of fura-2 AM was prepared by dissolving 50 µg of fura-2 AM in 20 µl of anhydrous DMSO, and the stock was stored at -5°C until needed. G-1 was obtained from Caymen Chemical (Ann Arbor, MI). A G-1 stock solution of 0.01 M was made in anhydrous DMSO, and aliquots of the stock solution were stored at -20°C . All remaining chemicals were purchased from Sigma Aldrich (Oakville, ON). A 0.01 M stock solution of 17β-estradiol was made in anhydrous DMSO and stored at room

temperature. Isoproterenol was combined with water to make a 0.001 M stock solution, which was stored at -20°C.

IX. Statistical Analysis

Data were analyzed using Clampfit 8.2 (Molecular Devices, Sunnyvale, CA). Sigma Plot 12.0 (Systat Software, Inc., Point Richmond, CA) software was used to graph data, fit concentration-response curves, and perform statistical analyses. Background fluorescence was subtracted from fluorescence measurements at each wavelength and measurements were then converted to an emission ratio (340/380 nm) using Felix software (PTI). The ratios were converted to Ca^{2+} concentrations using an *in vitro* calibration Ca^{2+} curve fit to a linear equation ($y = 334.8x – 116.3$). Myocyte contraction was measured as the difference between resting cell length and cell length at the peak of contraction. Ca^{2+} transients were measured as the difference between diastolic (baseline) Ca^{2+} and systolic (peak) Ca^{2+}. Data are presented as mean ± SEM. Differences between data obtained at each of the four estrous stages were analyzed using a *t*-test, a one-way ANOVA, or a two-way repeated measured (RM) ANOVA. Multiple comparisons were performed using a Holm-Sidak or a Fisher Least Significant Difference post-hoc test. Differences were reported as significant if $p < 0.05$.

Chapter 3: Results

I. Characterization of Estrous Cycle in Group-housed Female C57BL/6 Mice.

Vaginal smears were collected from sexually mature, female C57BL/6 mice (~ 3 mos) for a period of 14 days and their estrous cycles were tracked depending upon vaginal cytology, as described in the methods section. Figure 3.1 panels A and B show two representative examples of estrous stages observed over a 14-day period in young female mice that were housed in groups of 5. These females were not exposed to bedding collected from cages housing male mice. These female mice showed no regular progression through estrous stages. These non-cycling females remained in one estrous stage for a number of days. Moreover, rather than progressing through the estrous stages cyclically, these females often reverted back to a previous estrous stage. The irregularities in their cycling were similar to the estrous stages tracked in aged female mice (Fig. 3.1C and D).

In order to induce regular cycling, female mice were exposed to bedding collected from cages housing sexually-mature male mice. This bedding was placed in the females' cages daily for a period of seven days followed by 14 days of vaginal smear collection. Figure 3.2 A-D shows four representative examples of estrous stages observed over a 14-day period in young female mice exposed to male urine-soaked bedding. Group-housed females exposed to bedding collected from cages housing male mice exhibited regular estrous cycling. Additionally, the Whitten effect was also observed in several of the group housed females, as some cycle synchronicity was observed during the 14-day period (Whitten, 1966). Taken

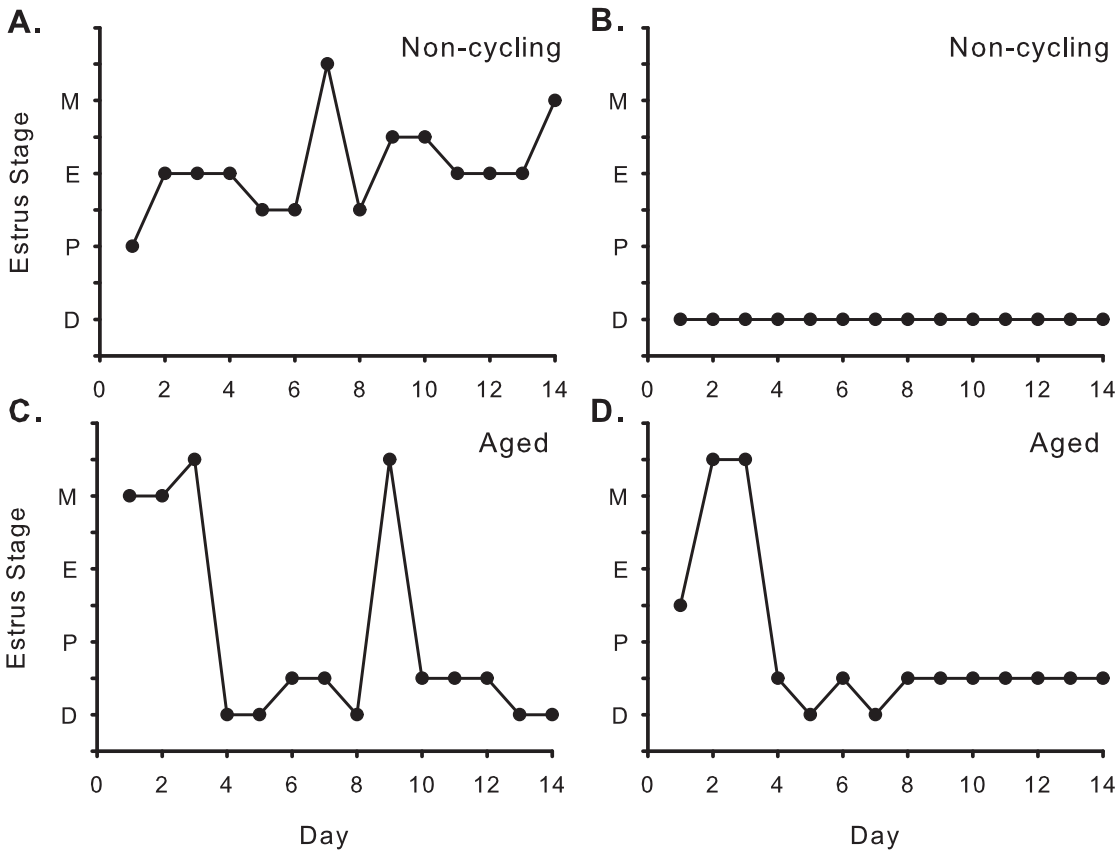


Figure 3.1. Group-housed female mice displayed irregular estrous cycles similar to those of aged female mice. Females were swabbed daily for a period of 14 days. The mouse's estrous stage was determined by vaginal cytology (Fig. 2.1). **A and B:** The estrous cycle was tracked in females that were not exposed to bedding collected from cages housing male mice. These mice showed irregular estrous cycling characterized by repetitive stages and reversion to previous stages. **C and D:** The estrous cycle was tracked in aged females. Cycle irregularities were similar to those observed in non-cycling young females. Abbreviations: D - Diestrus, P - Proestrus, E - Estrus, and M - Metestrus (n= 7 non-cycling mice and n = 9 aged mice).

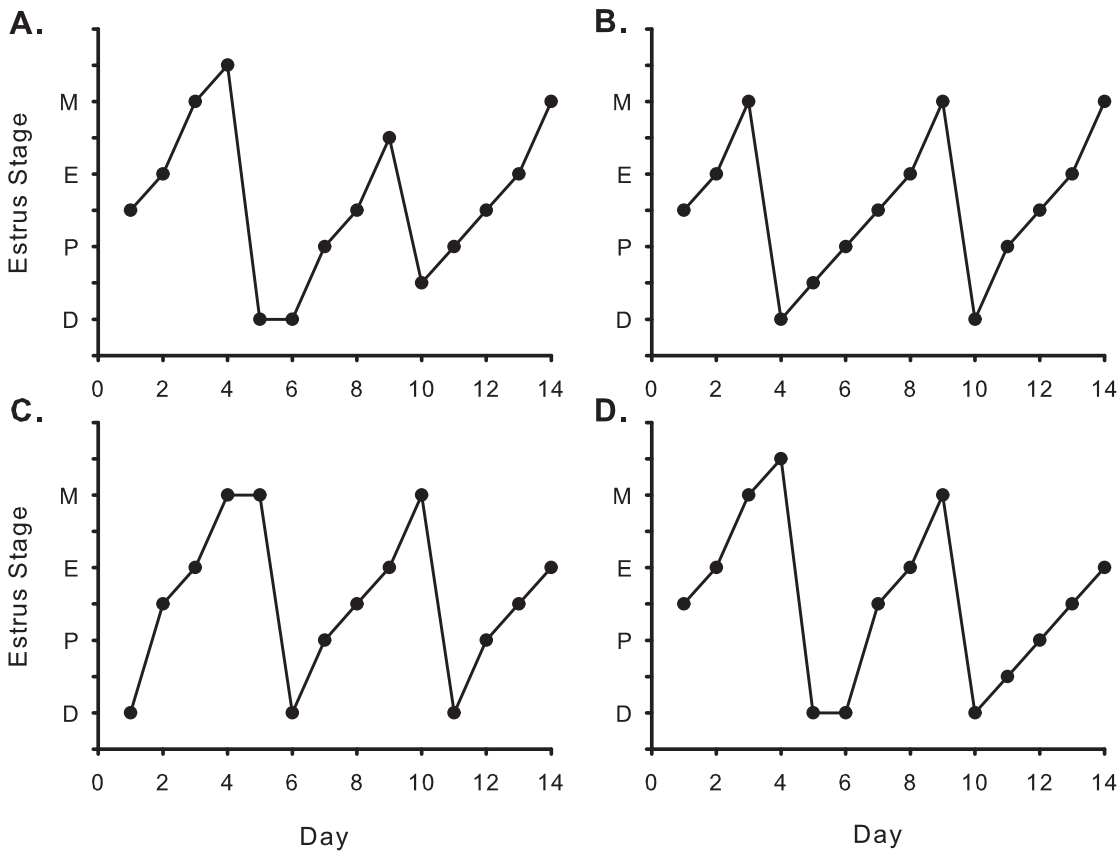


Figure 3.2. Group-housed female mice were induced to cycle through exposure to bedding collected from cages housing male mice. Females were exposed to bedding collected from cages housing sexually mature male mice. This bedding was added to their cages daily for 7 days prior to initial smear collection. Mice were then swabbed daily for a period of 14 days. The mouse's estrous stage was determined by vaginal cytology (Fig. 2.1). **A, B, C, and D:** Regular estrous cycling was routinely observed in females exposed to male urine-soaked bedding. Some cycle synchrony is visible among these group-housed females. Abbreviations: D - Diestrus, P - Proestrus, E - Estrus, and M - Metestrus (n= 8 cycling mice).

together, these results suggest that group-housed female mice do not exhibit regular estrous cycles unless induced to cycle through exposure to male pheromones.

II. Contractile Responses and Ca^{2+} Concentrations in Ventricular Myocytes Isolated From Females in the Four Stages of the Estrous Cycle.

To determine whether myocyte contractile function fluctuated with the estrous cycle, contractions and Ca^{2+} transients were measured in myocytes isolated from the hearts of mice in the four stages of the estrous cycle. Figure 3.3 A-D show representative examples of contractions (bottom) and Ca^{2+} transients (top) recorded from isolated myocytes paced at 4 Hz. Contraction amplitudes were similar in myocytes isolated from mice in diestrus, proestrus, and metestrus. However, the contraction was noticeably larger and faster in the myocyte isolated from the mouse in estrus. Ca^{2+} transient amplitudes were also similar in myocytes isolated from mice in diestrus, proestrus, and metestrus. However, the peak Ca^{2+} transient was visibly larger in the myocyte isolated from the mouse in estrus.

Mean data (Fig. 3.4 and 3.5) were consistent with the differences observed in representative contractions and Ca^{2+} transient amplitudes. Figure 3.4 shows contractile function in myocytes isolated throughout the estrous cycle. Contractions were normalized to resting cell length to determine fractional shortening. Fractional shortening was significantly larger in cells isolated from mice in estrus when compared to myocytes isolated during the three other stages of the estrous cycle (Fig. 3.4A). The velocity of shortening (Fig. 3.4B) and the velocity of lengthening (Fig. 3.4C) were also significantly faster in estrus when compared to diestrus, proestrus, and metestrus. These data show that ventricular myocytes

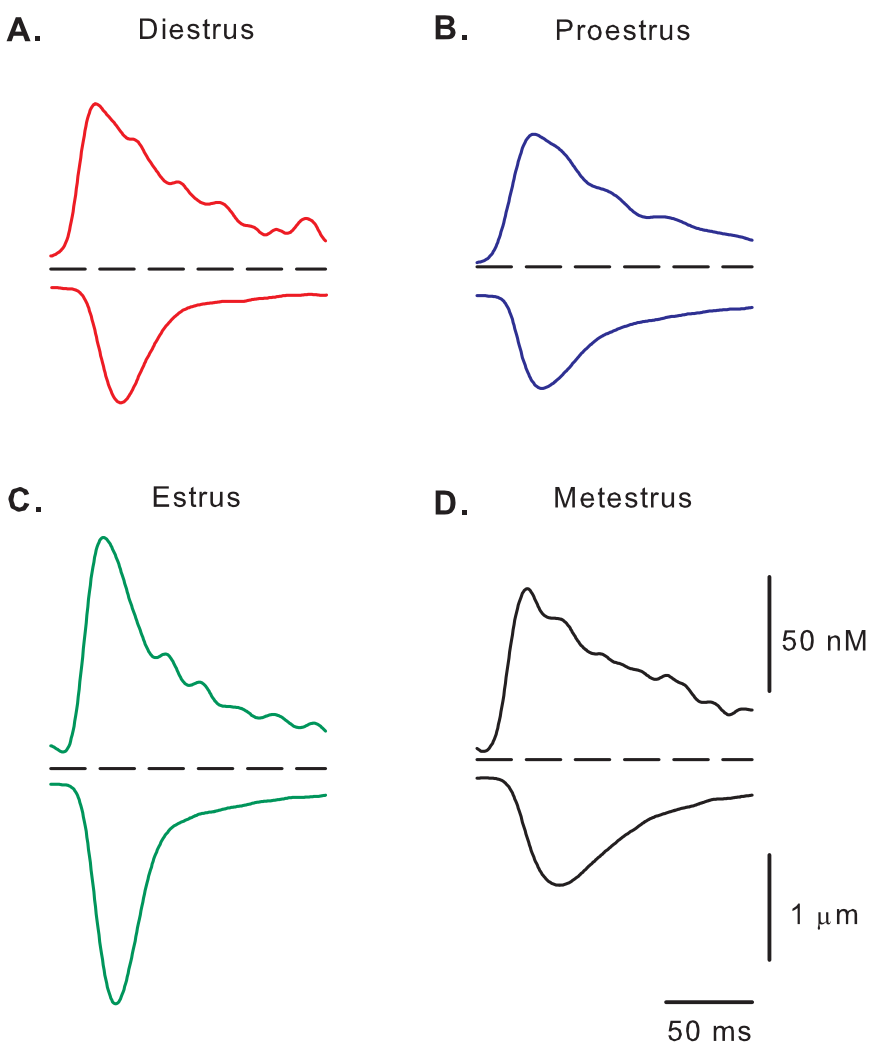


Figure 3.3. Representative examples of contractions and Ca^{2+} transients recorded in myocytes isolated from the hearts of mice in the four stages of the estrous cycle. Representative examples of contractions (bottom) and Ca^{2+} transients (top) recorded in field stimulated myocytes isolated from the hearts of mice in diestrus (**A**), proestrus (**B**), estrus (**C**), and metestrus (**D**) paced at 4 Hz.

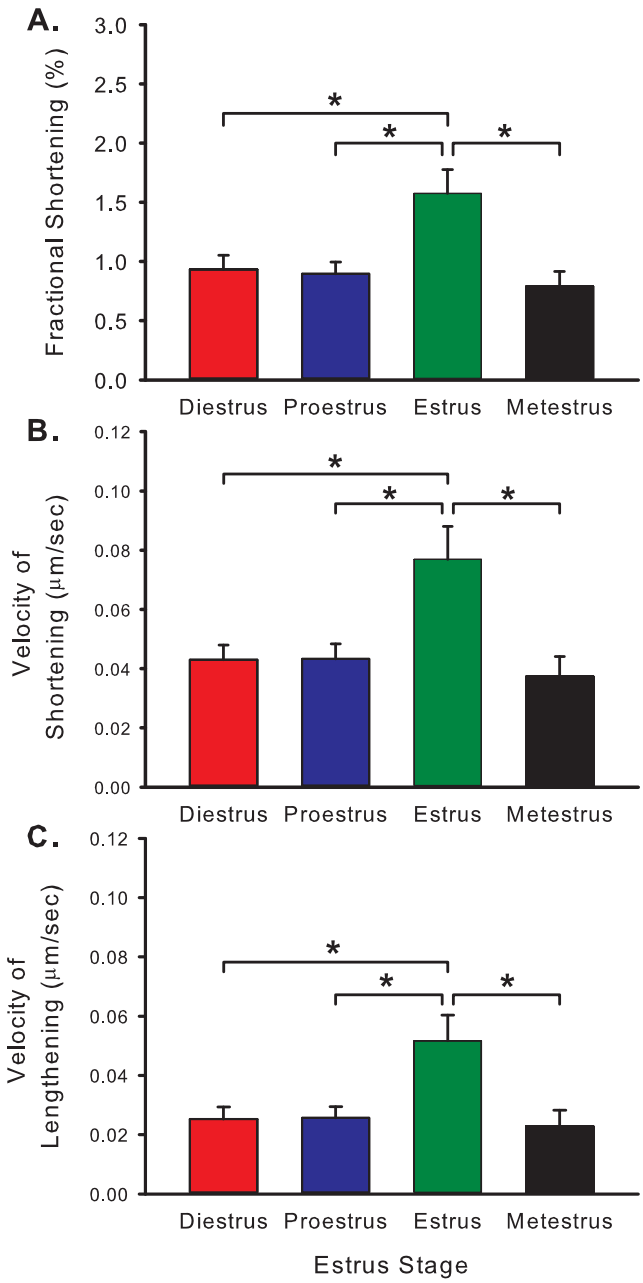


Figure 3.4. Fractional shortening was largest, and velocities of shortening and lengthening were fastest in estrus when compared to the other stages of the estrous cycle. Myocytes isolated from mice in the four stages of the estrous cycle were field stimulated at 4 Hz (37°C). **A:** Fractional shortening was significantly larger in estrus when compared to the other stages of the estrous cycle. **B:** Velocity of shortening was significantly faster in estrus when compared to the other stages of the estrous cycle. **C:** Velocity of lengthening was significantly faster in estrus when compared to diestrus, proestrus, and metestrus (n= 25 for diestrus, n = 27 for proestrus, n = 28 for estrus, n = 26 for metestrus; *$p<0.05$, One-way ANOVA).

isolated from mice in estrus have significantly larger and faster contractions than myocytes isolated from mice in the other stages of the estrous cycle.

Ca^{2+} homeostasis was also examined in myocytes isolated during the four stages of the estrous cycle. Diastolic Ca^{2+} levels did not fluctuate with the estrous cycle (Fig. 3.5A). There were no significant differences in systolic Ca^{2+} levels measured in myocytes isolated during the four stages of the estrous cycle (Fig. 3.5B). However, Ca^{2+} transient amplitudes were significantly larger in estrus compared to the other estrous stages (Fig. 3.5C). Velocities to peak, 50% decay, and 90% decay of the Ca^{2+} transient were also measured (Fig. 3.6A-C). However, these velocities did not fluctuate with the estrous cycle in myocytes paced at 4 Hz. Together, these data indicate that intracellular Ca^{2+} levels are significantly higher in estrus compared to the other estrus stages.

III. Contractile Responses and Ca^{2+} Concentrations In Rapidly Paced Cardiomyocytes From Females in the Four Stages of the Estrous Cycle.

To determine whether cardiac contractile function fluctuated with the estrous cycle at pacing rates that were closer to the physiological heart rate of 433 bpm for C57BL/6 mice, cells were stimulated at 6 Hz (Hoit et al., 2002). Contractions and Ca^{2+} transients were measured in myocytes from hearts isolated from mice in the four stages of the estrous cycle. Figure 3.7A-D shows representative cardiomyocyte contractions (bottom) and Ca^{2+} transients (top) from cells at all four stages of the estrous cycle. Contraction amplitudes were visibly larger in estrus and smaller in diestrus and metestrus. Ca^{2+} transient amplitudes

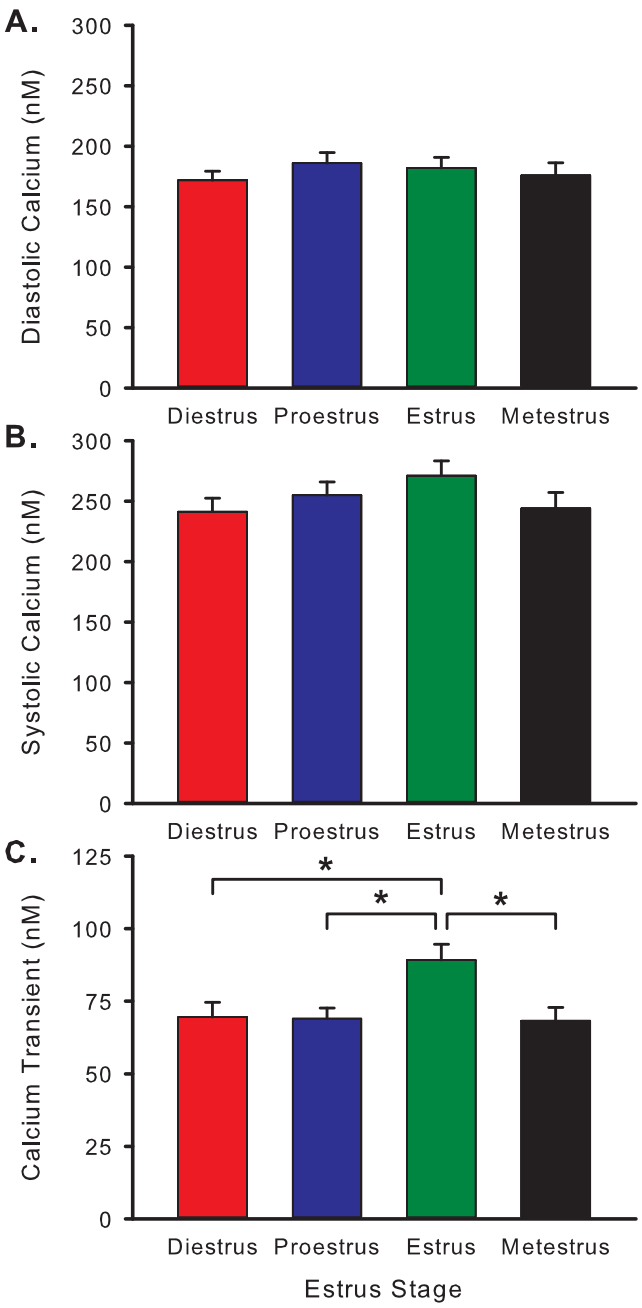


Figure 3.5. Peak Ca^{2+} transients were largest in myocytes isolated from mice in estrus compared to the other stages of the estrous cycle. Myocytes isolated from mice in the four stages of the estrus cycle were field stimulated at 4 Hz (37°C). **A:** Diastolic Ca^{2+} levels did not differ with the estrous cycle. **B:** Systolic Ca^{2+} levels were not significantly different depending upon the estrous stage of the mouse. **C:** Ca^{2+} transient amplitude was significantly higher in estrus when compared to the other three stages of the estrous cycle (n= 26 for diestrus, n = 25 for proestrus, n = 27 for estrus, n = 17 for metestrus; *$p<0.05$, One-way ANOVA).

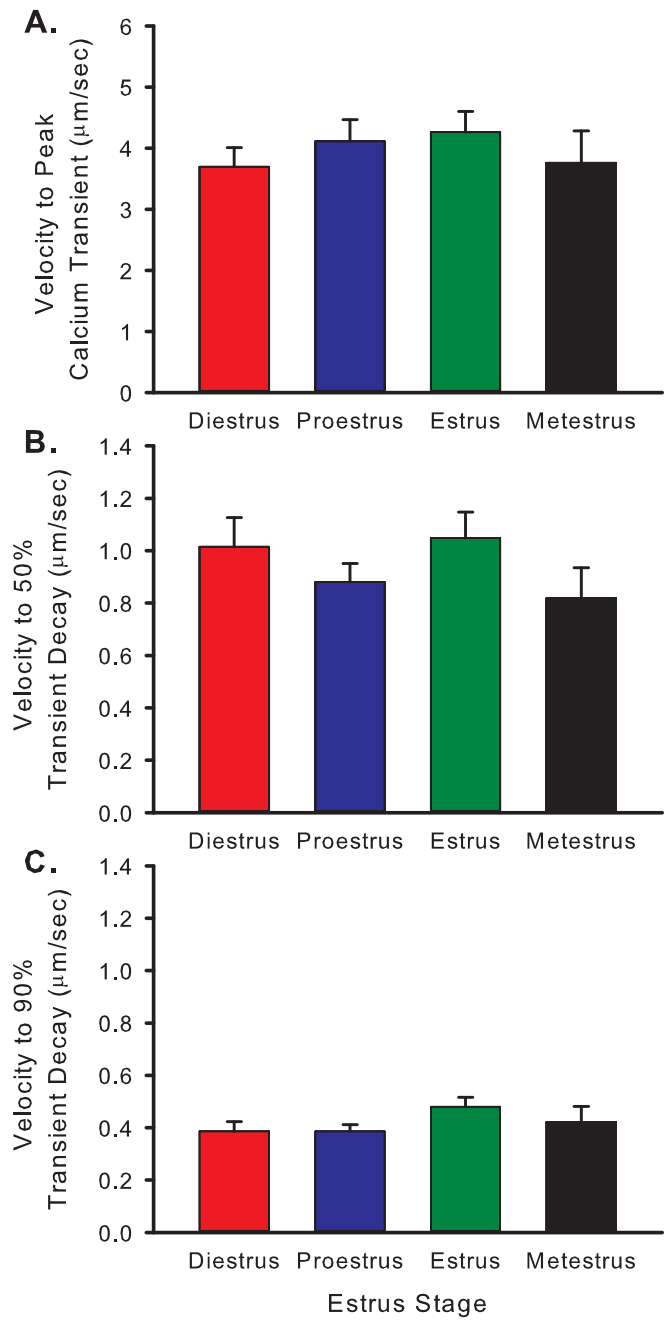


Figure 3.6. No significant differences were observed in the velocities to peak or decay of Ca^{2+} transients when cells were paced at 4 Hz. A, B, and C: Velocities to peak and 50% and 90% Ca^{2+} transient decay did not differ with the estrous cycle (n= 26 for diestrus, n = 25 for proestrus, n = 27 for estrus, n = 17 for metestrus; One-way ANOVA).

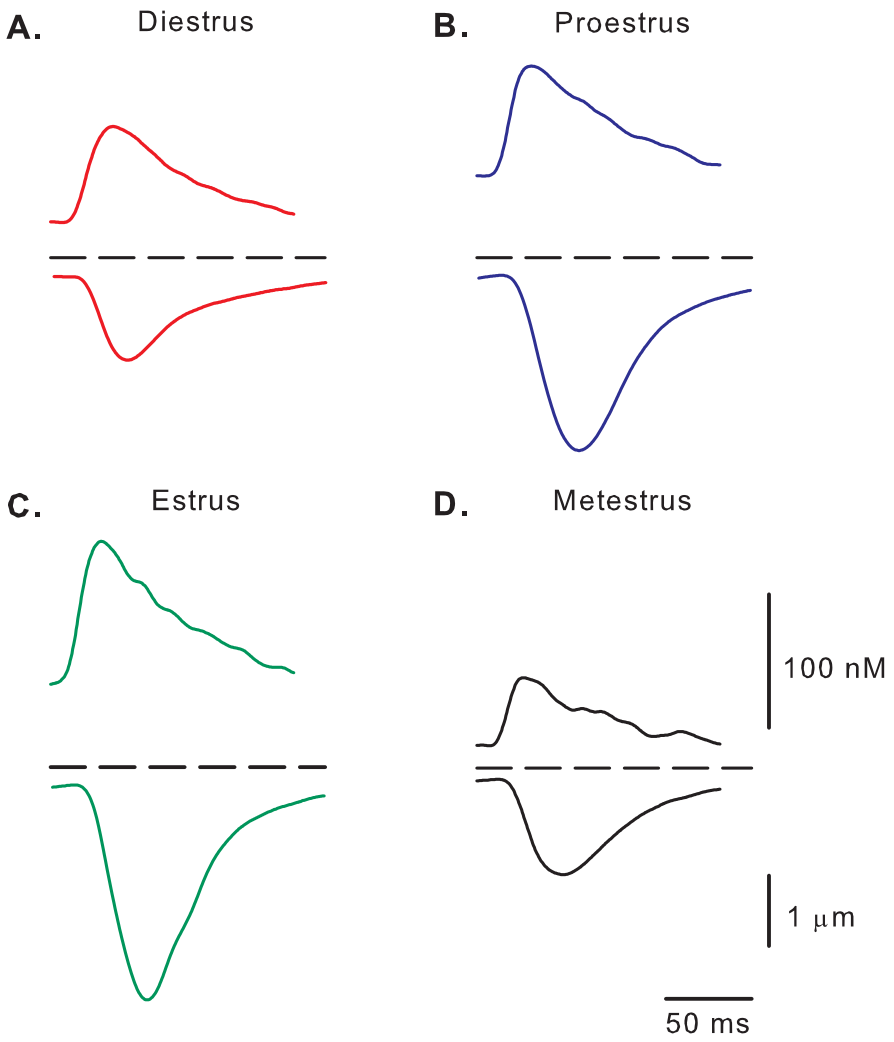


Figure 3.7. Representative examples of contractions and Ca^{2+} transients recorded in myocytes isolated from the hearts of mice in the four stages of the estrous cycle paced at a physiological frequency. Representative examples of contractions (bottom) and Ca^{2+} transients (top) recorded from cardiomyocytes isolated from the hearts of mice in diestrus (**A**), proestrus (**B**), estrus (**C**), and metestrus (**D**) paced at 6 Hz.

also appeared larger in estrus, but were similar in diestrus, proestrus, and metestrus.

Figure 3.8 shows mean contraction data in myocytes isolated throughout the estrous cycle. Fractional shortening was significantly larger in cells isolated from mice in estrus when compared to diestrus and metestrus (Fig. 3.8A) when cells were paced at 6 Hz. As well, the velocity of shortening (Fig. 3.8B) and the velocity of lengthening (Fig. 3.8C) were significantly faster in estrus when compared to both diestrus and metestrus. These data indicate that contractions remained larger and faster in estrus when compared to diestrus and metestrus when cells were paced at a higher frequency.

Ca^{2+} concentrations were also compared in myocytes subjected to physiological pacing rates. These studies showed that diastolic Ca^{2+} levels were significantly higher in proestrus and estrus when compared to metestrus (Fig. 3.9A), and systolic Ca^{2+} levels were highest in estrus when compared to metestrus (Fig. 3.9B). However, while Ca^{2+} transient amplitude was largest in estrus, this effect was not statistically significant. Velocities to peak, 50% decay, and 90% decay of the Ca^{2+} transient were also measured (Fig. 3.10A-C). While all three measurements were largest in estrus, none of these differences were statistically significant. Taken together, these observations showed that myocyte contractile function varied with the estrous cycle even when cells were paced at a physiological frequency.

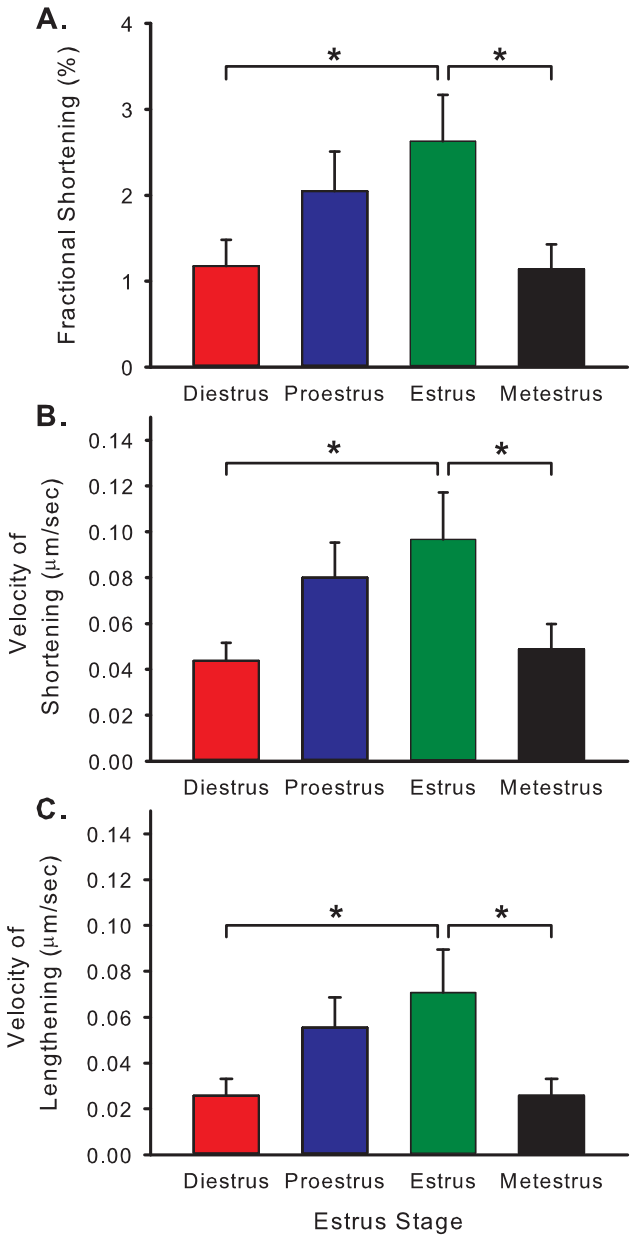


Figure 3.8. When subjected to pacing at 6 Hz, fractional shortening remained largest, and velocities of shortening and lengthening remained fastest in estrus when compared to the other stages of the estrous cycle. Myocytes isolated from mice in the four stages of the estrous cycle were field stimulated at 6 Hz (37°C). **A:** Fractional shortening was significantly larger in estrus when compared to diestrus and metestrus. **B:** Velocity of shortening remained significantly faster in estrus when compared to diestrus and metestrus. **C:** Velocity of lengthening remained significantly faster in estrus when compared to diestrus and metestrus (n= 12 for diestrus, n = 13 for proestrus, n = 12 for estrus, n = 12 for metestrus; *p<0.05, One-way ANOVA).

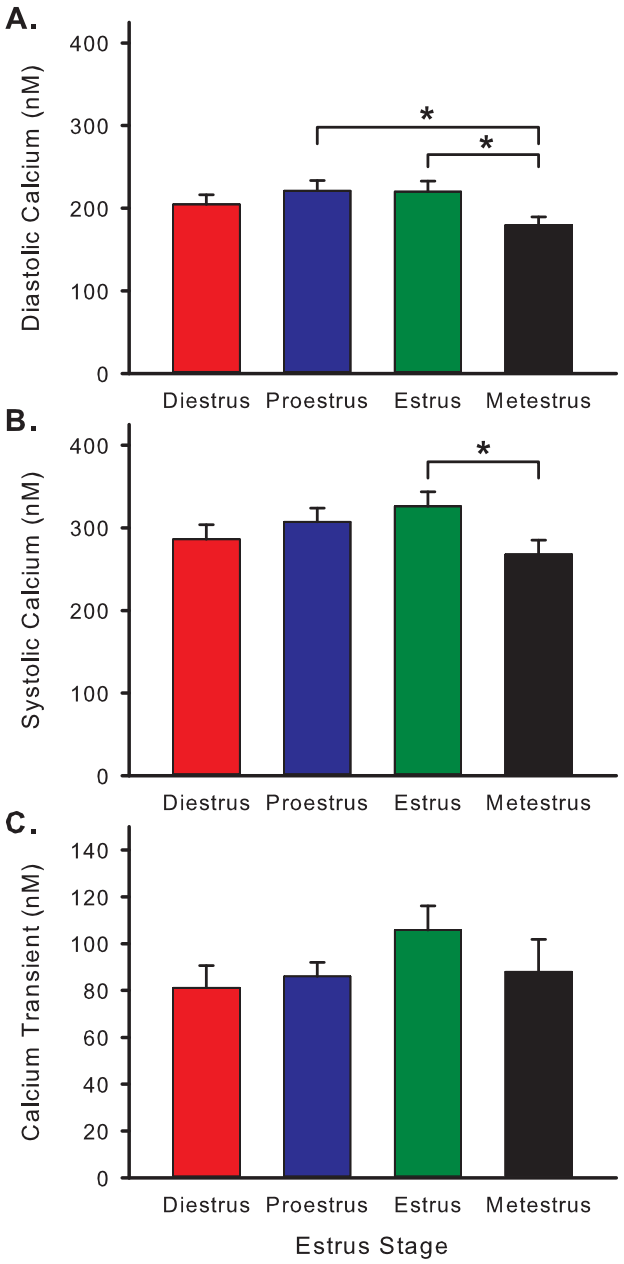


Figure 3.9. When subjected to pacing at 6 Hz, systolic Ca^{2+} levels were largest in myocytes isolated from mice in estrus compared to the other stages of the estrous cycle. Myocytes isolated from mice in the four stages of the estrous cycle were field stimulated at 6 Hz (37°C). **A:** Diastolic Ca^{2+} levels were significantly higher in proestrus and estrus when compared to metestrus. **B:** Systolic Ca^{2+} levels were significantly higher in proestrus and estrus when compared to metestrus. **C:** Differences in Ca^{2+} transients were abolished with rapid pacing (n= 13 for diestrus, n = 12 for proestrus, n = 12 for estrus, n = 7 for metestrus; *$p<0.05$, One-way ANOVA).

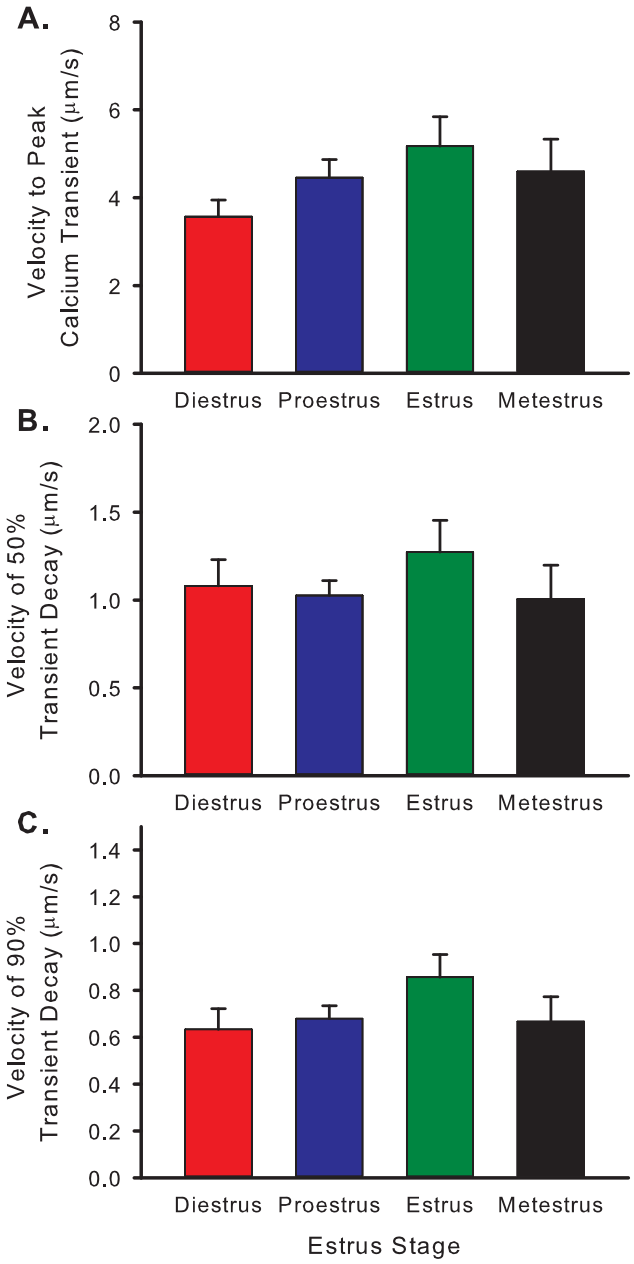


Figure 3.10. No significant differences were observed in the velocities to peak or decay of Ca^{2+} transients when cells were paced at 6 Hz. A, B, and C: Velocities to peak and 50% and 90% Ca^{2+} transient decay did not vary with the estrous cycle (n= 13 for diestrus, n = 12 for proestrus, n = 12 for estrus, n = 7 for metestrus; One-way ANOVA).

IV. Myofilament Ca^{2+} Sensitivity Measured in the Hearts from Mice in the Four Stages of the Estrous Cycle.

To determine whether myofilament Ca^{2+} sensitivity was affected by the estrous cycle, phase-loop plots were created in which intracellular Ca^{2+} levels were plotted against normalized fractional shortening throughout contraction and relaxation of the myocyte. The Ca^{2+} concentration during relaxation of the myocyte provides insight into myofilament Ca^{2+} sensitivity as shown in a previous study (Mellor et al., 2012). A representative phase-loop plot for a myocyte isolated from a mouse in estrus shows a clear right-ward shift in the relaxation phase of the loop when compared to the other estrous stages (Fig. 3.11A). To quantify the results observed with the phase-loop plot, the intracellular Ca^{2+} concentration at 50% myocyte relaxation was measured at each estrous stage. Intracellular Ca^{2+} levels at 50% relaxation were highest in estrus when compared to the other three stages of the estrous cycle (Fig 3.11B). Intracellular Ca^{2+} levels were significantly higher in estrus and proestrus when compared to metestrus (Fig. 3.11B). This demonstrates that more Ca^{2+} was required to activate the myofilaments in myocytes from the hearts of mice in estrus when compared to any of the other estrous stages.

Myofilament Ca^{2+} sensitivity was also assessed directly using a Carter assay to measure actomyosin MgATPase activity (Fig. 3.12A). Neither the maximum actomyosin MgATPase activity (Fig. 3.12B) nor the Hill coefficient (Fig. 3.12C), which is an indicator of cooperativity, was found to be different among hearts isolated from mice in the four stages of the estrous cycle. However, normalized data showed a clear right-ward shift of the actomyosin MgATPase activity-Ca^{2+} curve in

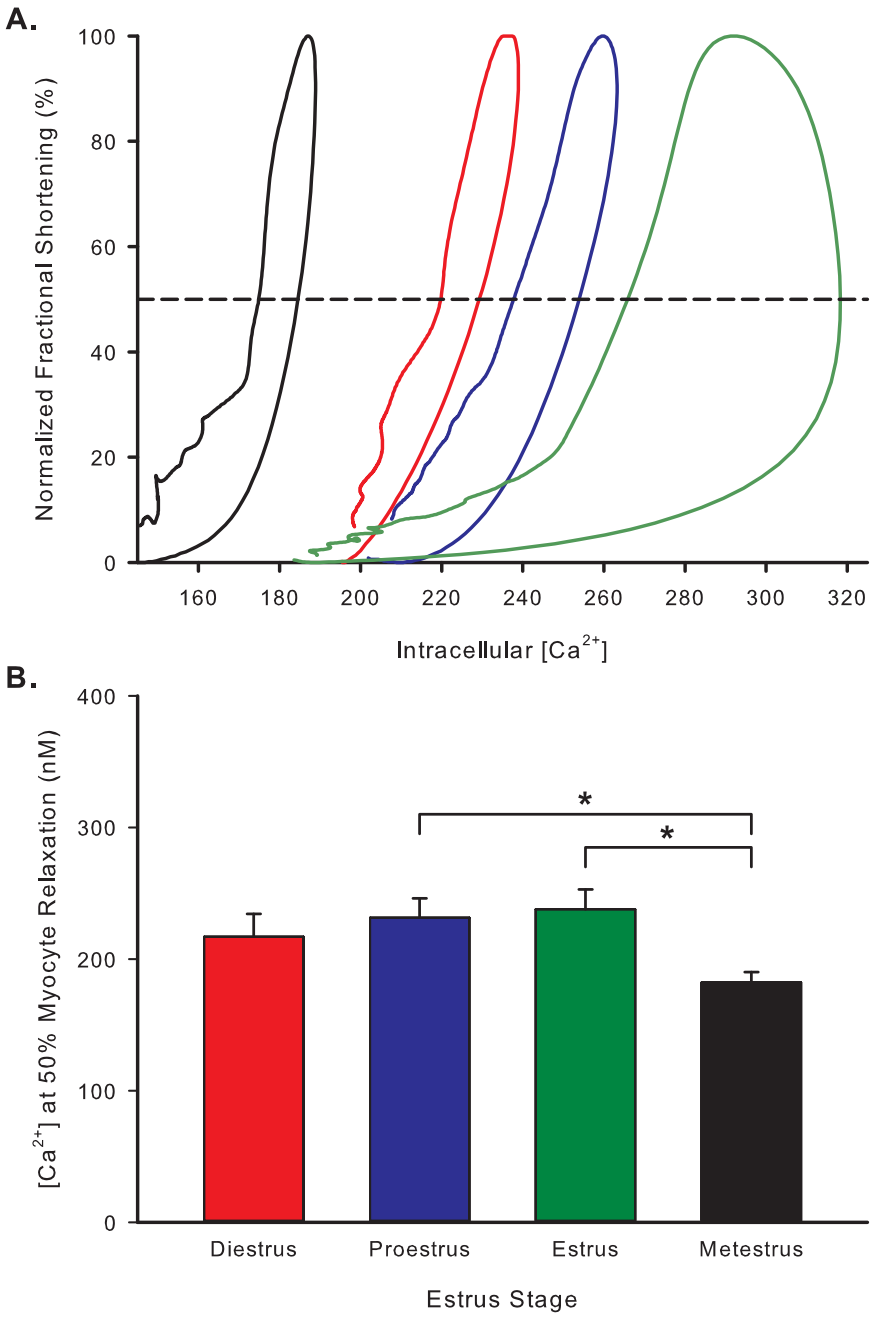


Figure 3.11. Cardiomyocytes isolated from mice in estrus display reduced myofilament responsiveness to Ca^{2+}. A: Representative phase-loops from myocytes isolated throughout the estrous cycle. A clear right-ward shift is visible for cells isolated during estrus and a clear left-ward shift is visible for hearts isolated during metestrus. **B:** Intracellular Ca^{2+} concentrations at 50% myocyte relaxation were significantly higher in estrus when compared to the other three stages of the estrous cycle (n= 13 for diestrus, n = 11 for proestrus, n = 12 for estrus, n = 10 for metestrus; *p<0.05, One-way ANOVA).

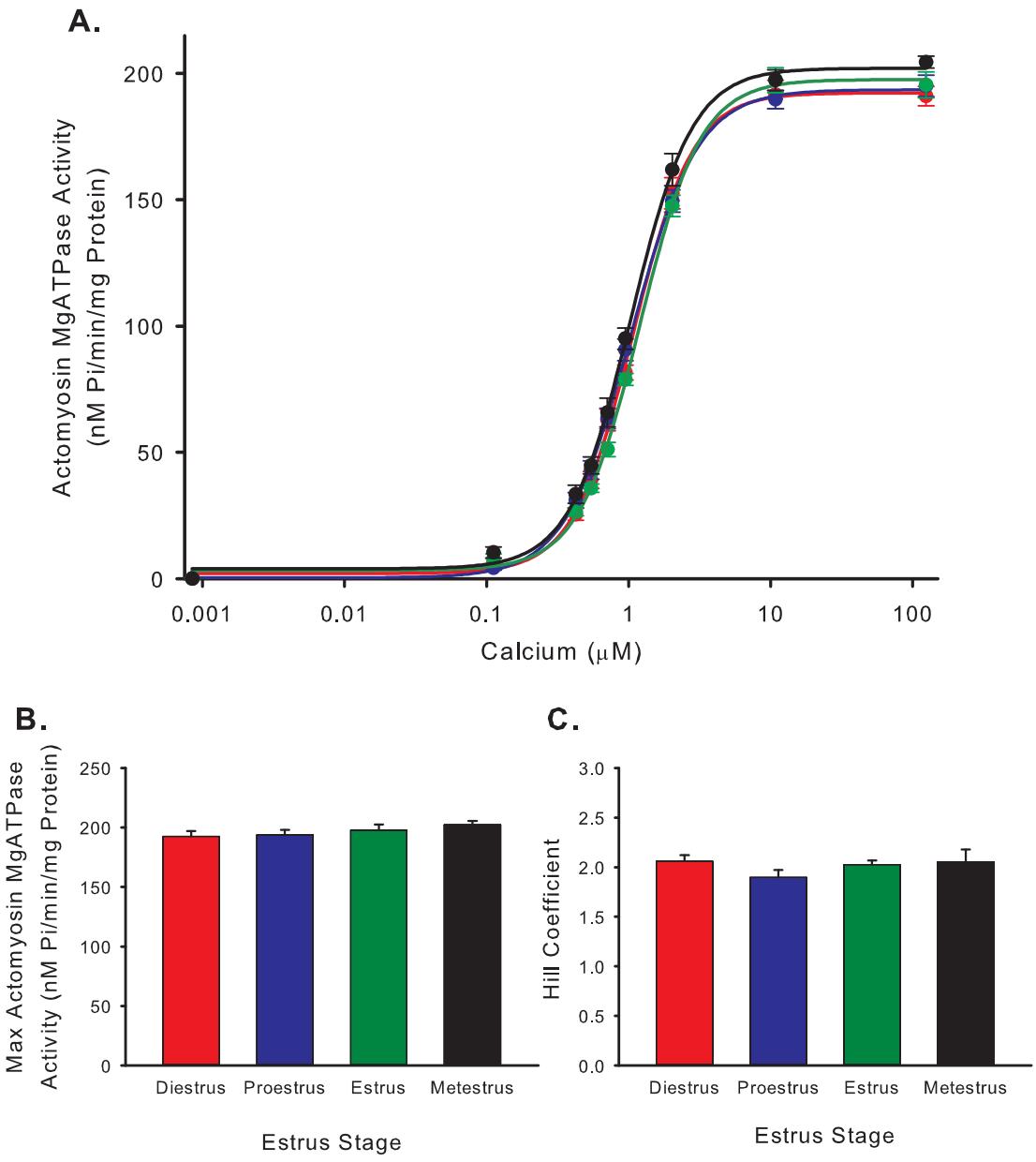


Figure 3.12. Maximum actomyosin MgATPase activity and the Hill coefficient did not differ depending upon the estrous stage. A: Actomyosin MgATPase activity in hearts isolated from mice in the four stages of the estrous cycle over a range of Ca^{2+} concentrations. **B:** Maximum actomyosin MgATPase activity was not affected by the estrous cycle. **D:** There were no differences observed in the Hill coefficients between any of the estrous stages (n= 7 hearts/group; One-way ANOVA).

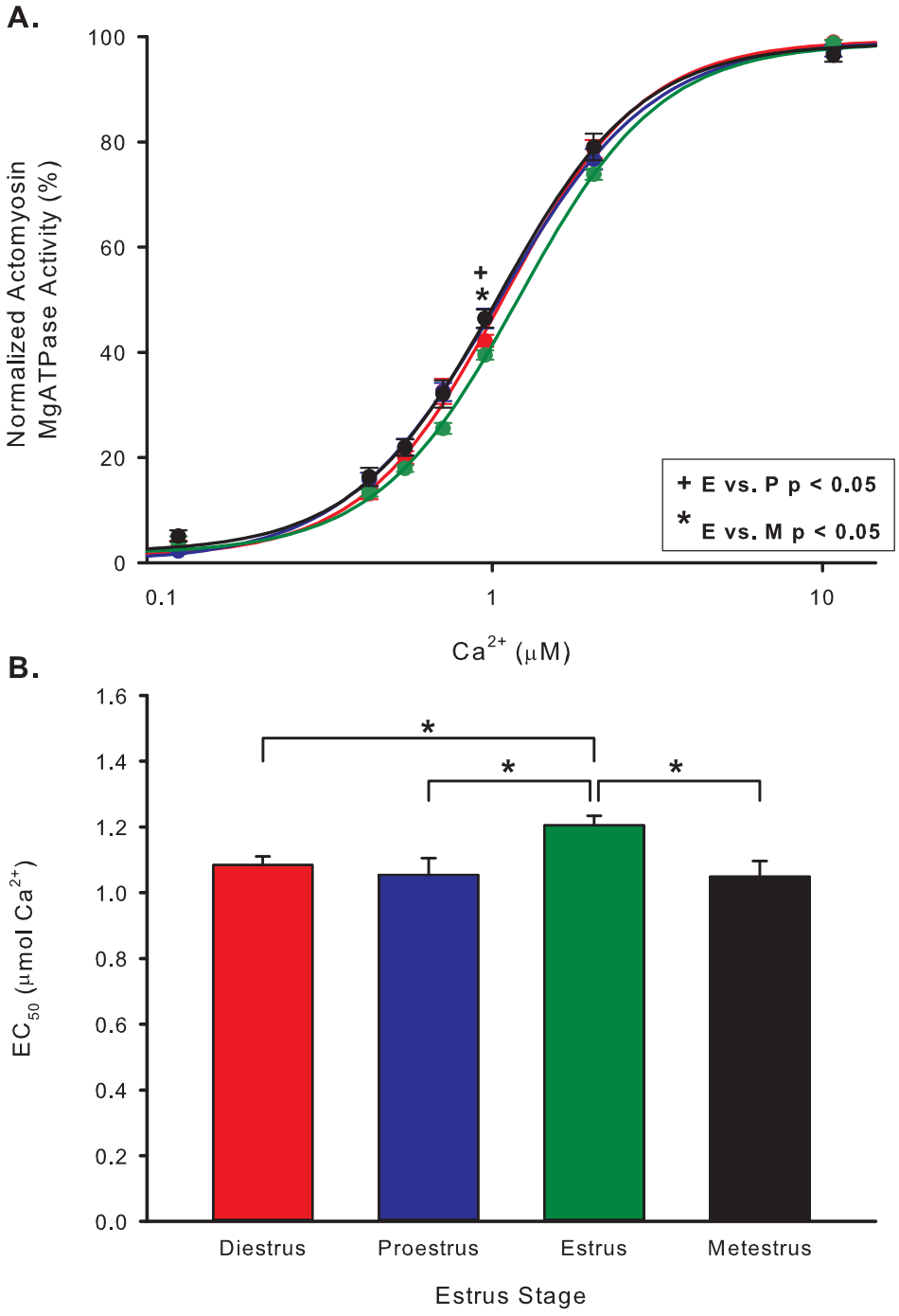


Figure 3.13. Myofilaments isolated from the hearts of mice in estrus are the least sensitive to Ca^{2+}. A: Normalized actomysin MgATPase activity indicates a clear right-ward shift in the curve for myofilaments from hearts that were isolated from mice in estrus when compared to the other estrus stages. **B:** The EC_{50} for Ca^{2+} was significantly larger in estrus when compared to the three other stages of the estrous cycle (n= 7 hearts/group; $^{+,*}p<0.05$, One-way ANOVA).

the hearts that were isolated from mice in estrus (Fig. 3.13A). This indicates that hearts isolated from mice in estrus have decreased myofilament Ca^{2+} sensitivity. Consistent with the right-ward shift of the actomyosin MgATPase-Ca^{2+} curve, the EC_{50} values for Ca^{2+} were significantly higher in hearts from mice in estrus when compared to the three other stages of the estrous cycle (Fig 3.13B). This indicates that myofilaments isolated from the hearts of mice in estrus were the least sensitive to Ca^{2+}.

V. Contractile Responses and Ca^{2+} Concentrations in ISO-treated Ventricular Myocytes From Females in the Four Stages of the Estrus Cycle.

To determine if β-adrenergic stimulation modified the effect of the estrous cycle on contractile function and Ca^{2+} concentrations, myocytes were exposed to the non-selective β-adrenergic agonist, isoproterenol. Serial dilutions from 1 nM to 1 μM were administered and cardiomyocyte contractions and Ca^{2+} transients were measured at each concentration. Figure 3.14 A-D illustrates representative examples of contractions (bottom) and Ca^{2+} transients (top) in the presence of 100 nM ISO in myocytes isolated from mice throughout the estrous cycle. Contraction amplitudes were largest in estrus and proestrus and smallest in diestrus and metestrus. Ca^{2+} transient amplitudes also were larger in both estrus and proestrus when compared to diestrus and metestrus. A concentration-response curve for fractional shortening was generated for a range of ISO concentrations (1 nM – 1μM) (Fig. 3.15). Concentrations of 10 nM ISO and greater resulted in significantly larger contractions in all four stages of the estrous cycle when the values were compared

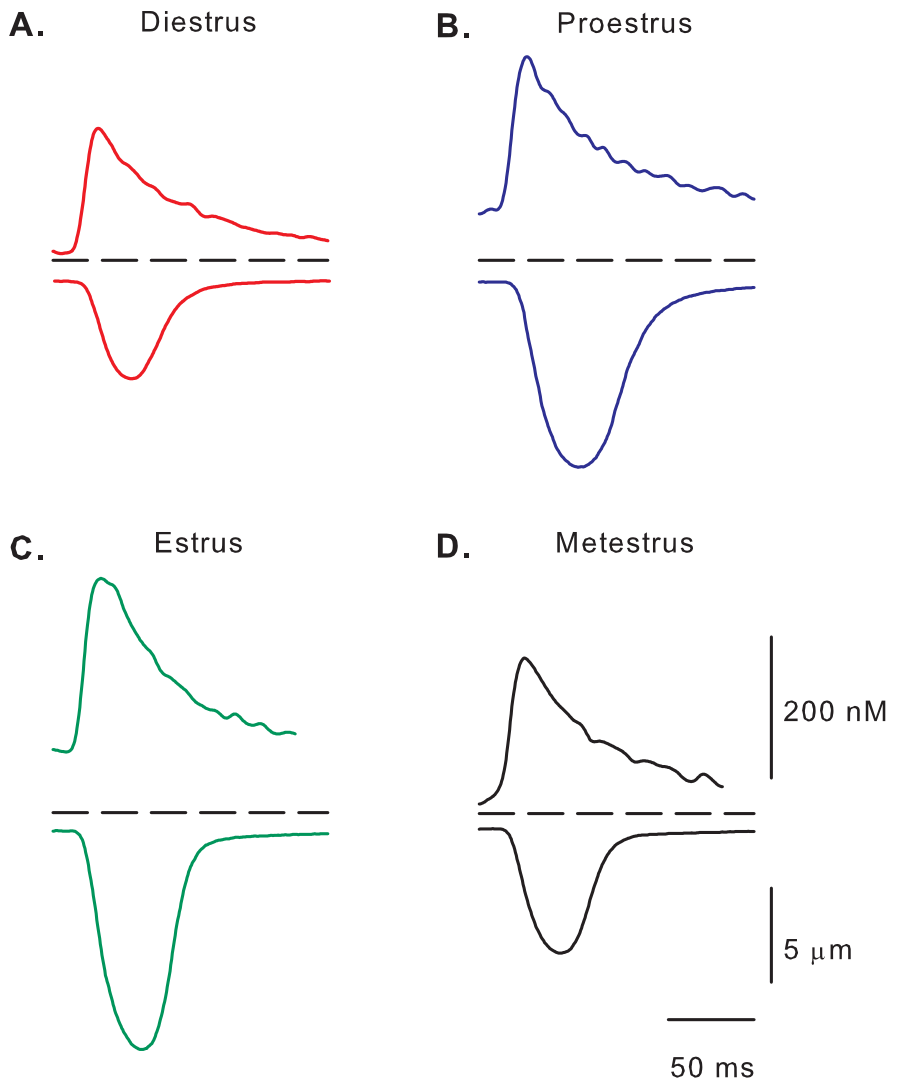


Figure 3.14. Representative examples of contractions and Ca^{2+} transients, in the presence of 100 nM isoproterenol, recorded in myocytes isolated from the hearts of mice in the four stages of the estrous cycle. Representative examples of contractions (bottom) and Ca^{2+} transients (top) recorded in field stimulated myocytes (4 Hz) isolated from the hearts of mice in diestrus (**A**), proestrus (**B**), estrus (**C**), and metestrus (**D**).

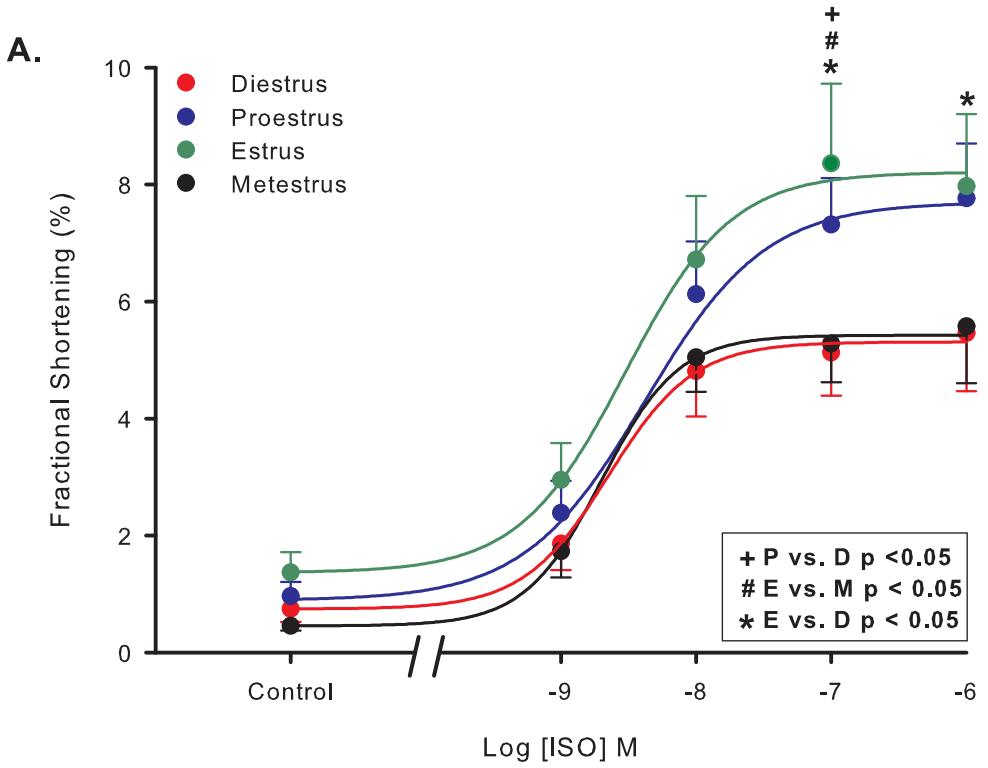


Figure 3.15. Fractional shortening remained largest in myocytes isolated from mice in estrus in the presence of ISO. Myocytes isolated from mice in the four stages of the estrous cycle were field stimulated at 4 Hz in the presence of ISO (37°C). **A:** Concentration-response curves for mice in the four stages of the estrous cycle for a range of ISO concentrations (n= 12 for diestrus, n = 13 for proestrus, n = 12 for estrus, n = 12 for metestrus; statistical significance as indicated, Two-way RM ANOVA).

to their respective controls. Additionally, in the presence of 100 nM ISO fractional shortening was significantly larger in estrus and proestrus when compared to diestrus. Fractional shortening was also significantly larger in estrus when compared to metestrus in the presence of 100 nM ISO. When the concentration of ISO was increased to 1 μM, responses were larger in estrus when compared to diestrus. By contrast, no difference in the EC_{50} values for ISO were observed throughout the estrous cycle (Fig. 3.16A and 3.16B).

A concentration-response curve was also generated for Ca^{2+} transient amplitudes over the same range of ISO concentrations (1 nM – 1μM) (3.17A). All ISO concentrations caused a significant increase in Ca^{2+} transient amplitudes in all four stages of the estrous cycle when compared to their respective controls. Further, Ca^{2+} transient amplitudes were significantly larger in estrus when compared to diestrus in the presence at concentrations from 1 nM to 1 μM ISO. Ca^{2+} transient amplitudes were also significantly larger in proestrus when compared to diestrus in the presence of 10 nM and 100 nM ISO. No difference in EC_{50} values for ISO were observed throughout the estrous cycle (Fig. 3.18A and 3.18B).

VI. Contractile Responses and Ca^{2+} Concentrations following Acute Application of 17β-estradiol or the GPER agonist, G-1, in Cardiomyocytes From Females in the Four Stages of the Estrous Cycle.

To determine whether acute application of the estrogen receptor agonist, 17β-estradiol, modulated cardiac contractile function in myocytes isolated from mice in the four stages of the estrous cycle, contractions and Ca^{2+} transients were measured in the presence of the drug. Two concentrations of 17β-estradiol were

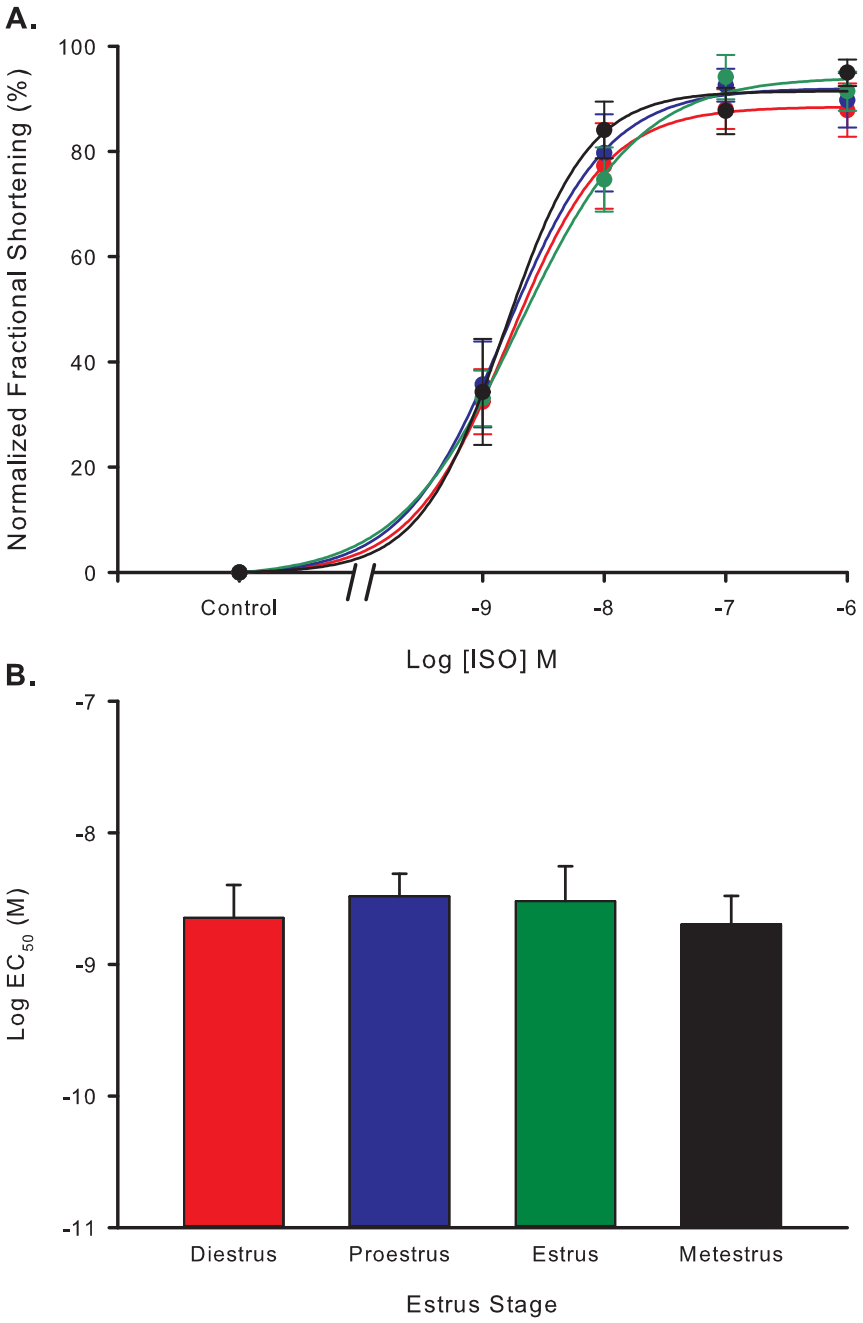


Figure 3.16. EC_{50} values for fractional shortening were similar at all stages of the estrous cycle. A: Normalized concentration-response curves for fractional shortening indicated no difference in EC_{50} values between the four stages of the estrous cycle. **B:** The EC_{50} values for ISO were not affected by the estrous cycle (n= 11 for diestrus, n = 12 for proestrus, n = 11 for estrus, n = 10 for metestrus; One-way ANOVA).

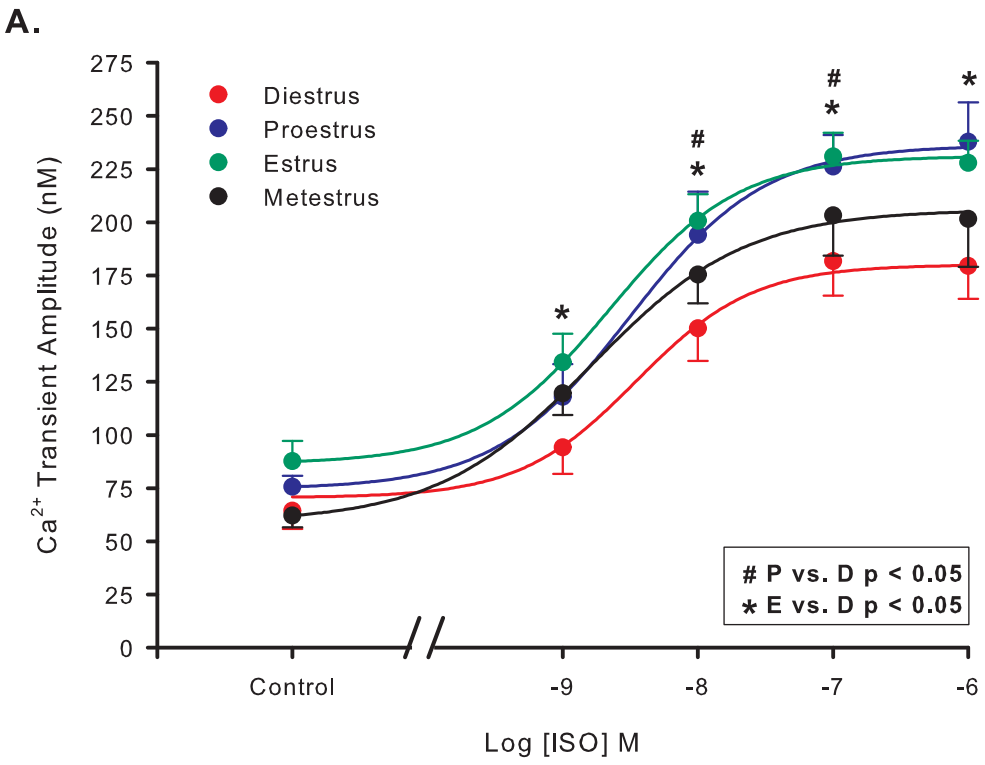


Figure 3.17. Ca^{2+} transient amplitudes were largest in myocytes isolated from mice in proestrus and estrus in the presence of ISO. Myocytes isolated from mice in the four stages of the estrous cycle were field stimulated at 4 Hz in the presence of ISO (37°C). **A:** Concentration-response curves for mice in the four stages of the estrous cycle over a range of concentrations of ISO (n= 12 for diestrus, n = 13 for proestrus, n = 12 for estrus, n = 12 for metestrus; statistical significance as indicated, Two-way RM ANOVA).

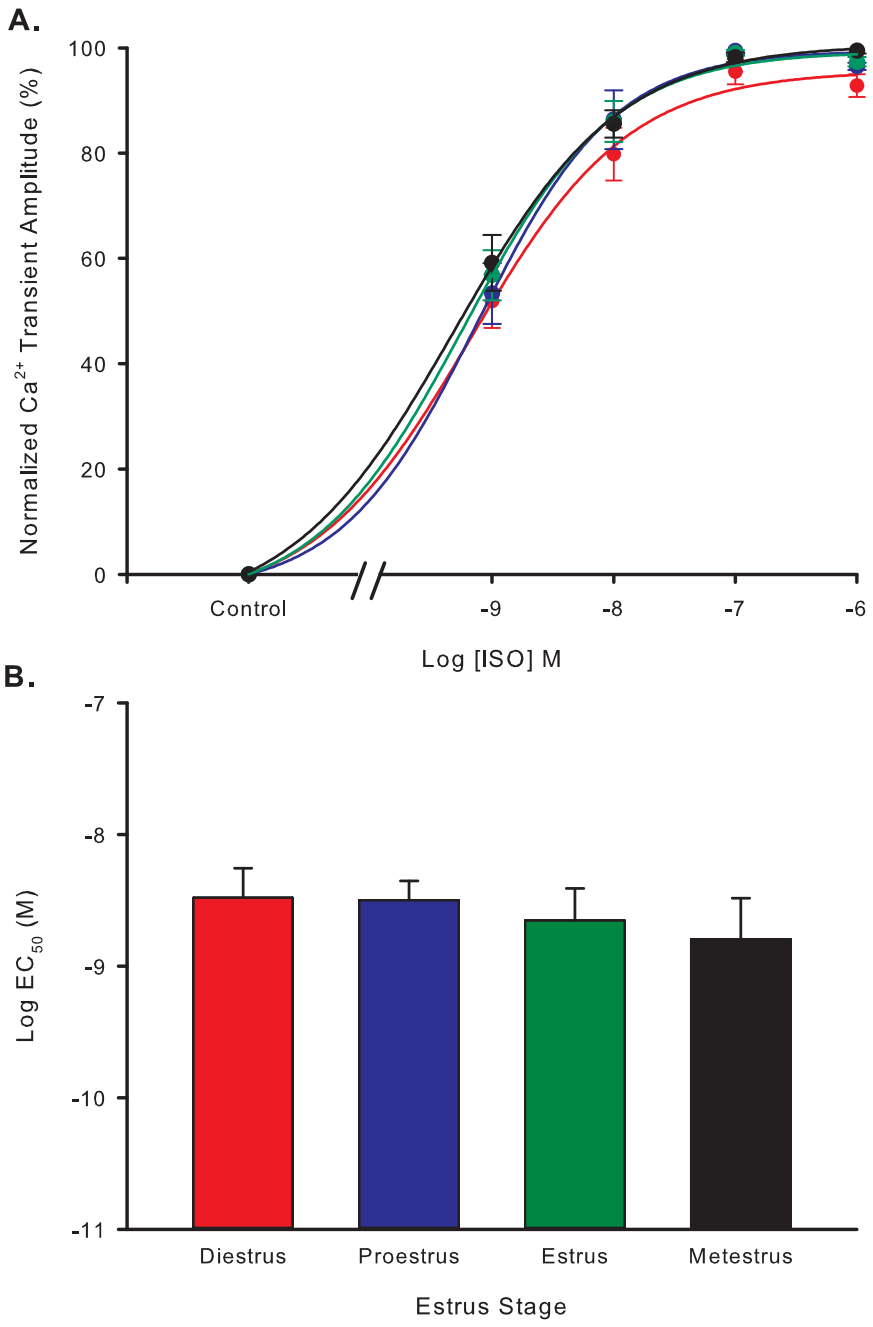


Figure 3.18. EC_{50} values for Ca^{2+} transient amplitude were similar at all stages of the estrous cycle. A: Normalized concentration-response curves for Ca^{2+} transient amplitude indicated no difference in EC_{50} values between the four stages of the estrous cycle. **B:** The EC_{50} values for ISO were not affected by the estrous cycle (n= 11 for diestrus, n = 12 for proestrus, n = 11 for estrus, n = 10 for metestrus; One-way ANOVA).

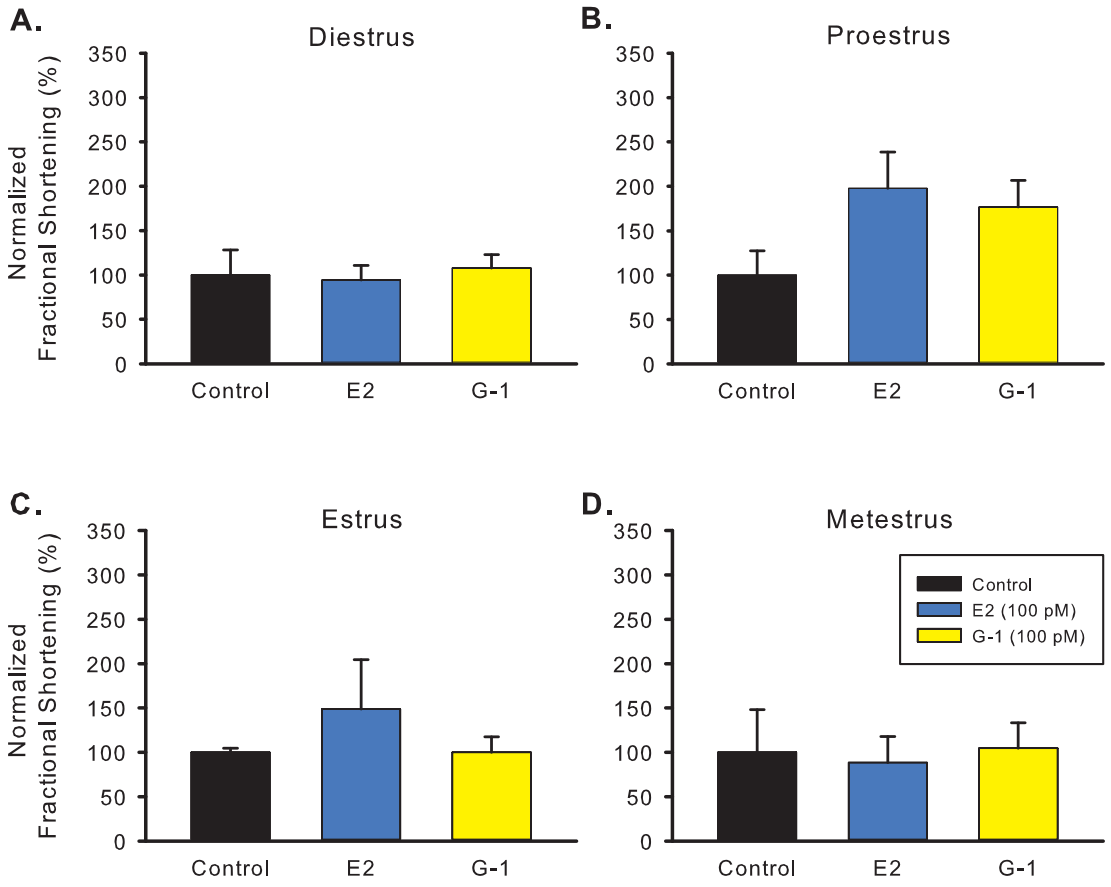
examined: a physiological concentration of 100 pM and a supra-physiological concentration of 100 nM. The activity of estradiol was confirmed by acutely applying 30 μM to myocytes isolated from female mice, as previously shown in guinea-pig myocytes (Jiang et al., 1992). Acute application of 30μM estradiol significantly increased Ca^{2+} transient amplitude (Control: 68.6 ± 7.0 nM and E2: 86.32 ± 4.3 nM, n = 5; $p < 0.05$) but had no effect on peak contractions (Control: 1.13 ± 0.3 % and E2: 1.21 ± 0.3 %, n = 5; $p < 0.05$).

In the presence of a physiological concentration (100 pM) of 17β-estradiol, peak fractional shortening was not statistically different from values measured in the absence of drug regardless of the estrous stage (Fig. 3.19A-D). Ca^{2+} transient amplitudes in myocytes from mice at all four stages of the estrous cycle were also not affected by 100 pM 17β-estradiol (Fig. 3.20A-D).

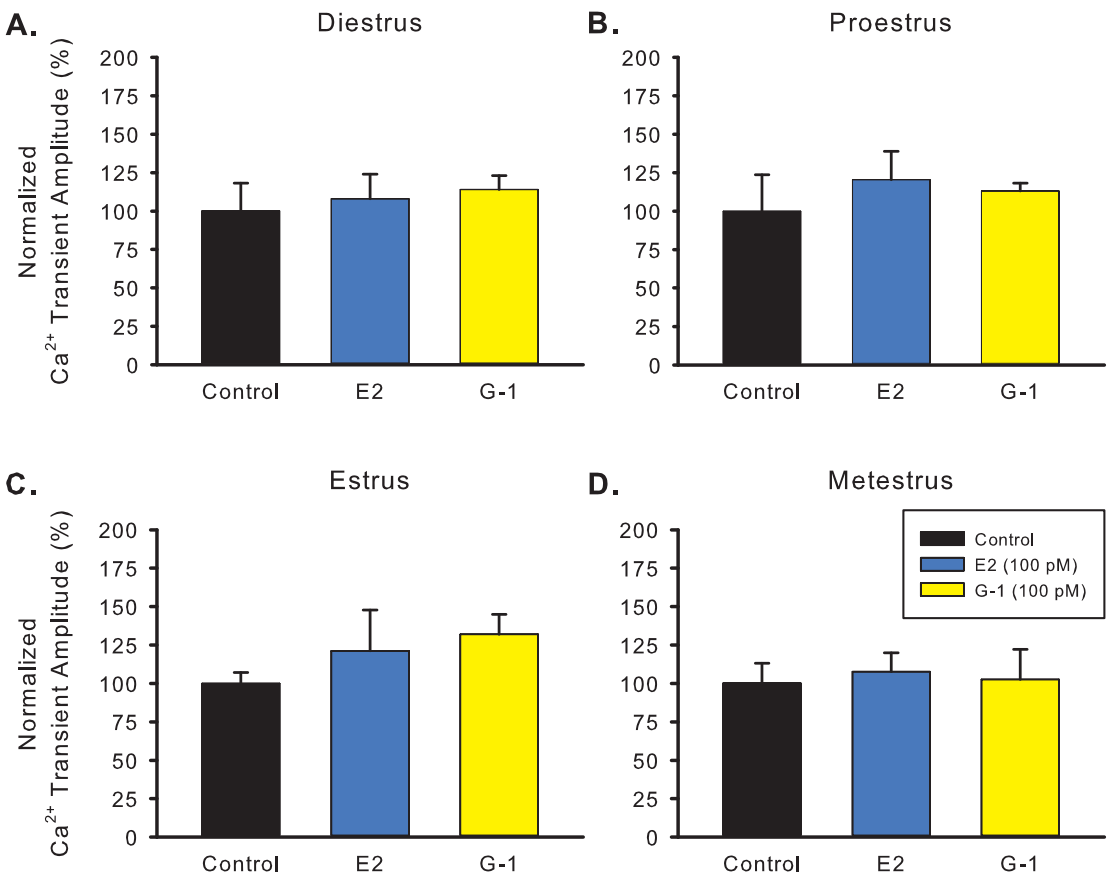
When cells isolated from mice throughout the estrous cycle were exposed to a supra-physiological concentration (100 nM) of 17β-estradiol, fractional shortening was also comparable to peak contractions measured in the absence of drug (3.21A-D). As well, Ca^{2+} transient amplitudes were not significantly different from control when measured in the presence of 100 nM 17β-estradiol (Fig. 3.22A-D). These observations demonstrate that both physiological and supra-physiological concentrations of 17β-estradiol did not affect cardiac contractile function and Ca^{2+} homeostasis in myocytes isolated during the four stages of the estrous cycle.

Contractions and Ca^{2+} transients were also measured in the presence of the GPER agonist, G-1, to determine if this agonist altered cardiac contractile function in myocytes isolated from mice throughout the estrous cycle. Contractions and Ca^{2+}

transients were recorded in the presence 100 pM and 100 nM G-1 (Figs. 3.19 - 3.22). In the presence of a 100 pM of G-1, no differences were observed in fractional shortening when compared to responses recorded in the presence of vehicle control at any stage of the estrous cycle (Fig. 3.19A-D). As well, Ca^{2+} transient amplitudes recorded in the same myocytes were unaffected by 100 pM G-1 (Fig. 3.20A-D). When the concentration of G-1 was increased to 100 nM, fractional shortening was also not affected by the drug at any stage of the estrous cycle (Fig. 3.21A-D). Further, Ca^{2+} transient amplitudes recorded in the presence of 100 nM G-1 were not statistically different from vehicle control in any of the estrous stages (Fig. 3.22A-D). Taken together, these results suggest that acute application of varying concentrations of G-1 had no effect on contractions or Ca^{2+} transients in myocytes isolated throughout the estrous cycle.



3.19. Fractional shortening was not affected by exposure to either 100 pM 17β-estradiol or 100 pM G-1 in myocytes isolated from mice in the four stages of the estrous cycle. A-D: Fractional shortening measured in myocytes isolated from mice throughout the estrous cycle was not affected by either E2 or G-1 when compared to control (n= 4 control, 6 E2, and 8 G-1 for diestrus, n = 5 control, 7 E2, and 5 G-1 for proestrus, n = 5 control, 3 E2, and 7 G-1 for estrus, n = 5 control, 7 E2, and 7 G-1 for metestrus; *t*-test).



3.20. Ca^{2+} transient amplitudes were not affected by exposure to 100 pM estradiol or 100 pM G-1 in myocytes isolated from mice in the four stages of the estrous cycle. A-D: Ca^{2+} transient amplitudes measured in myocytes isolated from mice in diestrus, proestrus, estrus, and metestrus were not affected by acute application of either E2 or G-1 (n= 4 control, 6 E2, and 8 G-1 for diestrus, n = 4 control, 7 E2, and 8 G-1 for proestrus, n = 5 control, 4 E2, and 6 G-1 for estrus, n = 4 control, 7 E2, and 5 G-1 for metestrus; *t*-test).

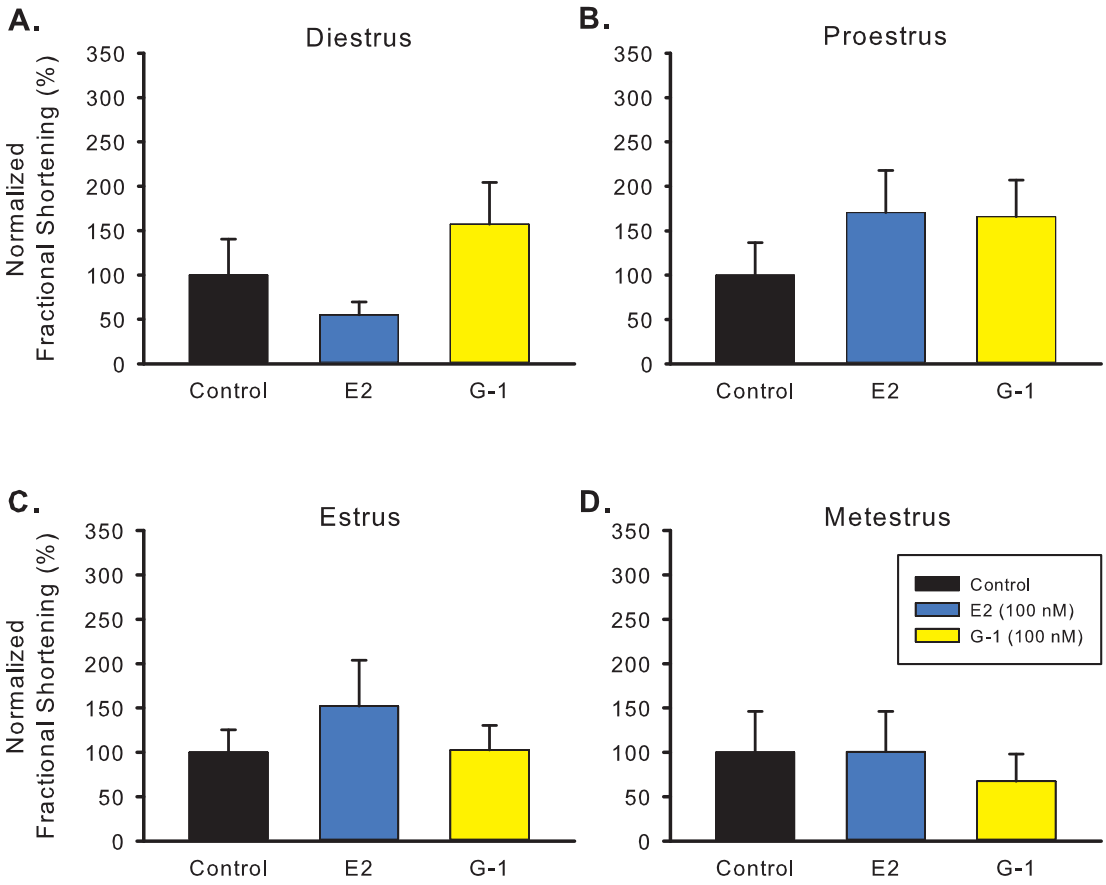


Figure 3.21. Higher concentrations of 17β-estradiol (100 nM) and G-1 (100 nM) had no effect on fractional shortening in myocytes isolated from mice in the four stages of the estrous cycle. A-D: Fractional shortening measured in myocytes isolated from mice in the four stages of the estrous cycle was not modified by acute application of E2 or G-1 (n= 5 control, 5 E2, and 8 G-1 for diestrus, n = 5 control, 7 E2, and 6 G-1 for proestrus, n = 5 control, 8 E2, and 8 G-1 for estrus, n = 5 control, 6 E2, and 6 G-1 for metestrus; *t*-test).

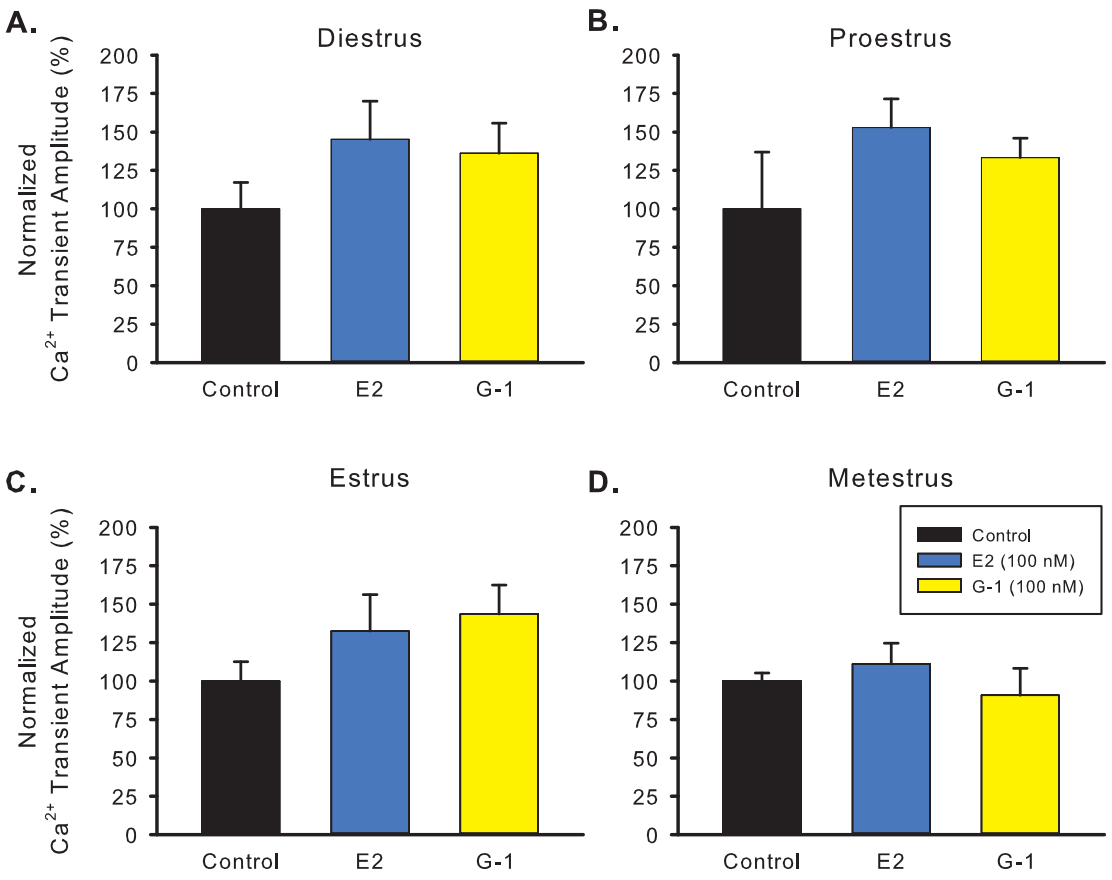


Figure 3.22. Ca^{2+} transient amplitudes recorded in myocytes isolated throughout the estrous cycle were unaffected by higher concentrations of 17β-estradiol (100 nM) and G-1 (100 nM). A-D: Acute application of high concentrations of either E2 or G-1 had no effect on Ca^{2+} transient amplitudes measured in myocytes isolated from mice in diestrus, proestrus, estrus, and metestrus. (n= 5 control, 5 E2, and 8 G-1 for diestrus, n = 4 control, 7 E2, and 8 G-1 for proestrus, n = 5 control, 8 E2, and 8 G-1 for estrus, n = 3 control, 7 E2, and 5 G-1 for metestrus; *t*-test).

Chapter 4: Discussion

I. Overview of Key Findings

The first objective of this study was to determine whether group-housed female mice displayed cyclical changes in their estrous stages. It was established that sexually mature, group-housed female mice showed infrequent and irregular changes in estrous stages. Thus, these females were deemed, "non-cycling". The irregularities observed in the cycling of these females were similar to the estrous cycles tracked in aged female mice. However, when sexually mature, group-housed female mice were exposed to bedding collected from cages housing male mice, regular cycling was observed. These findings show that group-housed female mice do not display cyclical estrous stages unless induced to cycle through exposure to bedding from cages housing male mice.

Another objective of this study was to determine if cardiac contractile function and Ca^{2+} concentrations fluctuated with the estrous cycle in myocytes isolated from mice in the four stages of the estrous cycle. Similar results were seen when myocytes were paced at either 4 or 6 Hz. Contractions were significantly larger in myocytes isolated from mice in estrus when compared to the other three stages of the estrous cycle. These myocytes also had more rapid velocities of shortening and lengthening. Ca^{2+} transient amplitudes were also significantly larger in estrus when compared to the other estrous stages. Thus, intracellular Ca^{2+} levels were significantly elevated in myocytes isolated from mice in estrus, and this effect of the estrous cycle was maintained when myocytes were paced at a physiological frequency.

This study also sought to determine whether myofilament Ca^{2+} sensitivity fluctuated with the estrous cycle. The results of both the phase-loop plots as well as the actomyosin MgATPase activity assay indicated that myofilaments isolated from mice in estrus were the least sensitive to Ca^{2+}. Intracellular Ca^{2+} concentrations at 50% myocyte relaxation were significantly higher in estrus, indicating that more Ca^{2+} was required to maintain contraction in cells from mice in estrus. Consistently, the EC_{50} of Ca^{2+} was found to be significantly higher in estrus when compared to the other estrous stages. Although contractions and Ca^{2+} transients were largest in estrus, these findings suggest that myofilament Ca^{2+} sensitivity actually decreased in this stage.

Another objective of this study was to determine if the response to β-AR stimulation fluctuated with the estrous cycle. This was assessed through the acute application of the non-selective β-adrenergic agonist, isoproterenol. Fractional shortening and Ca^{2+} transient amplitudes were largest in estrus and smallest in diestrus and metestrus over a range of ISO concentrations. No differences in EC_{50} values for ISO were observed throughout the estrous cycle. Thus, the effects of the estrous cycle on myocyte contractile function were maintained even in the presence of β-AR stimulation.

The final objective of the study was to determine if acute application of 17β-estradiol or G-1 modified contractile function in myocytes isolated from mice throughout estrous cycle. Interestingly, acute application of either a physiological or supra-physiological concentration of 17β-estradiol had no effect on contractions or Ca^{2+} transients. Additionally, acute application of G-1 had no effect on contractile

function or Ca^{2+} concentrations regardless of the estrous stage examined. Therefore, acute application of 17β-estradiol or G-1 did not modify contractile function in myocytes isolated from mice throughout the estrous cycle.

II. Characterization of Estrous Cycle in Group-housed Female C57BL/6 Mice.

i. Non-cycling, group-housed female mice

The results of this study showed that regular estrous cycling was suppressed in group-housed female mice. These findings are consistent with the results of previous studies that show that female pheromones can suppress the estrous cycle in female mice that are housed in groups (Whitten, 1959). Interestingly, the irregularities observed in the estrous stages in group-housed females were similar to the irregularities in cycling observed in 24 month-old female mice. The onset of irregular cycling due to age is thought to occur between 20 – 22 mos in C57BL/6 female mice (Boot et al., 1956). With increasing age, C57BL/6 mice exhibit irregular cycling rather than anestrus, which is distinguished by the absence of any identifiable estrous stage and occurs in some mouse strains (Boot et al., 1956). Irregular cycling is characterized by the recurrent presence of vaginal smears containing leukocytes and/or cornified cells. However, smears containing nucleated cells, which are characteristic of proestrus, are infrequent (Boot et al., 1956). Additionally, the estrous stages no longer appear cyclical but become repetitive and random (Boot et al., 1956). The results of the present study demonstrated that reproductive senescence was associated with irregular estrous cycles characterized

by recurrent diestrus/estrus smears. Moreover, similar irregular cycling was seen in young female mice that were housed in groups.

Interestingly, the prolonged presence of leukocytes and/or cornified cells documented in aged female C57BL/6 mice is indicative of the absence of ovulation in these mice (Allen, 1922). It is well known that ovulation is triggered by a rise in 17β-estradiol levels (Allen, 1922). Moreover, the rise in 17β-estradiol levels prior to ovulation results in the transition from a leukocyte-containing diestrus smear to a nucleated cell-containing proestrus smear. Therefore, the absence of the pre-ovulatory rise in 17β-estradiol explains why proestrus smears are rarely observed in these aged female mice. As group-housed female mice also rarely exhibited proestrus smears, these results suggest that these females may also be experiencing an absence of the pre-ovulatory rise in 17β-estradiol that triggers ovulation. Thus, female mice housed in groups would be expected to have more constant basal levels of estrogen rather than the fluctuating estrogen levels that are characteristic of the estrous cycle (Butcher et al., 1974).

ii. Induced cycling in group-housed female mice

Regular, 4 – 6 day estrous cycles were observed in group-housed females after they were exposed to bedding collected from cages housing male mice. This finding is consistent with the results of previous studies that show that male pheromones are capable of inducing regular estrous cycles in group-housed female mice (Whitten, 1966). Male urine is thought to be the main source of the pheromones that induce estrous cycling (Whitten, 1956). As well, induction of the

estrous cycle using male urine is thought to be androgen-dependent, as urine collected from castrated males does not induce estrous cycling (Bruce, 1965). The pheromones that are contained within male urine are referred to as "primer pheromones", indicating that they take time to exert their effects (Ma et al., 1999). The observation that male pheromones require time before any effects are seen is consistent with the data in the present study, as 5 - 7 days were required before regular estrous cycling was observed in females exposed to male pheromones.

Taken together, these results suggest that it is possible to study female mice in two contexts. In the first context, "non-cycling" group-housed females could be used in order to investigate physiological processes in the absence of the estrous cycle. The second context would be to investigate sexually mature "cycling" female mice. The estrous cycle is analogous to the human menstrual cycle; therefore, this model would more closely approximate the physiology of a sexually mature female human. There is a sex bias when it comes to using animals in research (Beery and Zucker, 2011). Females are most frequently disregarded because the assumption is made that results obtained from males apply to females. In addition, many researchers use male animals in order to avoid the complication of the estrous cycle. The results of the present study suggest that such concerns may be unfounded.

III. Contractile Responses and Ca^{2+} Concentrations In Ventricular Myocytes Isolated From Females in the Four Stages of the Estrous Cycle.

To date, only a small number of studies have investigated the effect of the rodent estrous cycle on cardiovascular function. Thus, relatively little is known about the effects the estrous cycle may have on overall cardiovascular function.

The results of this study showed that contractions were significantly larger in myocytes isolated from mice in estrus when compared to the other stages of the estrous cycle. This may be due to fluctuations in plasma estradiol levels, which are characteristic of the estrous cycle (Butcher et al., 1974). Estrus has been previously reported as the estrous stage with the lowest plasma estradiol levels (Saito et al., 2009; Walmer et al., 1992). Therefore, these results suggest that low estradiol levels may be responsible for the increased contraction size seen in estrus. These findings are compatible with the results of previous studies in OVX mice. Fractional shortening is significantly larger in myocytes isolated from OVX mice compared to sham-operated controls (Curl et al., 2003). Furthermore, estrogen replacement reverses this observed increase in contraction (Curl et al., 2003). Taken together, these findings suggest that cardiac contractions are augmented by conditions associated with low estradiol levels, such as the estrus stage of the estrous cycle or ovariectomy.

As contractions are activated by the release of Ca^{2+} from the SR (Bers, 2002), larger contractions in estrus could be due to increased Ca^{2+} transient amplitudes. Indeed, the present study showed that Ca^{2+} transient amplitudes were significantly larger in estrus when compared to the three other stages of the estrous cycle. By contrast, no differences were observed in diastolic Ca^{2+} levels. This suggests that at rest, myocytes isolated during the four stages of the estrous cycle had similar levels of intracellular Ca^{2+}. These findings suggest that one or more components of the EC coupling pathway may be modulated by the estrous cycle.

Previous studies have shown that Ca^{2+} release from the SR is proportional to the amount of incoming $I_{Ca,L}$ (Bers, 2002; Delbridge et al., 1996). Therefore, the increased Ca^{2+} transient amplitude in estrus may be due to an increase in the magnitude of $I_{Ca,L}$. Interestingly, L-type Ca^{2+} channel expression is enhanced in mice lacking both nuclear ERs (ERα and ERβ) (Johnson et al., 1997). This finding suggests that estrogen may regulate L-type Ca^{2+} channel expression through activation of the nuclear ERs. It is likely that less activation of the nuclear ERs occurs when estrogen levels are low. Therefore, L-type Ca^{2+} channel expression, and in turn $I_{Ca,L}$, may be increased when estradiol levels fall during estrus.

Another component of the EC coupling pathway that may be affected by estrogen is the SR. Previous studies have shown that OVX increases both Ca^{2+} transient amplitude (Curl et al., 2003) and SR Ca^{2+} stores (Fares et al., 2012; Kravtsov et al., 2007). Therefore, changes in estrogen levels throughout the estrous cycle may affect SR Ca^{2+} stores. A large amount of Ca^{2+} within the SR may increase the availability of stored Ca^{2+} for release (Bers, 2002). Therefore, the larger Ca^{2+} transient amplitudes seen in estrus may be due, in part, to increased SR Ca^{2+} stores, which would give rise to enhanced SR Ca^{2+} release.

Another factor that may contribute to the increased Ca^{2+} transient amplitude observed in myocytes from estrus is altered ventricular repolarization. Saito et al. showed that myocytes isolated from mice in estrus had reduced $I_{to,f}$ and $I_{K,slow}$, which both play key roles in repolarization (Saito et al., 2009). These reduced K^+ currents result in a significant increase in APD50 and APD90 in myocytes isolated from mice in estrus when compared to the other stages of the estrous cycle (Saito et al., 2009).

An increase in APD50 and APD90 could increase Ca^{2+} influx into the cardiomyocyte. Therefore, these findings provide an additional mechanism by which intracellular Ca^{2+} levels may be increased in low estrogen conditions.

No changes were observed in the velocities to peak or decay of the Ca^{2+} transients in myocytes isolated from mice throughout the estrous cycle. The velocity of Ca^{2+} transient decay is due to the activity of both SERCA2a and NCX, which contribute to 92% and 7% of the removal of Ca^{2+} from the cytosol, respectively (Hove-Madsen and Bers, 1993). Additionally, phospholamban (PLB) also influences the speed of Ca^{2+} transient decay by inhibiting SERCA (Bers, 2002). Phosphorylation of PLB by PKA relieves the inhibition of SERCA and causes a more rapid Ca^{2+} transient decay. As no differences were observed in the velocity of Ca^{2+} transient decay, these results suggest that expression levels of SERCA2a, NCX, and PLB were not altered by the estrous cycle.

Cardiac contractile function was also assessed at a more physiological pacing frequency in myocytes isolated throughout the estrous cycle. The resting heart rate for C57BL/6 mice is close to 6 Hz (Hoit et al., 2002). Therefore, pacing cells at 6 Hz more closely approximates physiological heart rate. Contractions and Ca^{2+} transients remained larger in myocytes isolated from mice in estrus when compared to the other stages of the estrous cycle when cells were paced at 6 Hz. It is well established that increased pacing frequencies lead to enhanced contraction (Koch-Weser and Blinks, 1963). Thus, it is likely that the increased fractional shortening seen with physiological pacing at 6 Hz is due to the increased Ca^{2+} release caused by increases in peak $I_{Ca,L}$ and increased SR Ca^{2+} content (Bers, 2002). Taken together,

these results indicate that the effects of the estrous cycle on cardiac contractile function are present even when cells are paced at a more physiological frequency.

In summary, the present study showed that myocytes isolated from mice in estrus have significantly larger fractional shortening than myocytes isolated from mice in the other estrous stages. The larger contractions observed in estrus are likely due to the increased Ca^{2+} transient amplitudes seen in these myocytes. The increased Ca^{2+} release from the SR may be due to increased peak $I_{Ca,L}$ along with increased SR Ca^{2+} stores, and decreased K^{+} repolarizing currents. Additional experiments are required to test these ideas.

IV. Myofilament Ca^{2+} Sensitivity Measured in the Hearts from Mice in the Four Stages of the Estrous Cycle.

The cardiac myofilaments function as the translation site where an increase in intracellular Ca^{2+} is converted into mechanical cellular contraction (Bers, 2002). As mentioned previously, the sensitivity of the myofilaments to Ca^{2+} plays an integral role in cardiomyocyte contraction. This study showed that myofilament Ca^{2+} sensitivity was lowest in myocytes and hearts isolated from mice in estrus compared to the other stages of the estrous cycle. Previous studies have used phase-loop plots to provide an estimate of myofilament Ca^{2+} sensitivity (Mellor et al., 2012; Spurgeon et al., 1990). The relaxation portion of the loop is thought to provide an indication of the relationship between contraction decay and $[Ca^{2+}]_i$. This relationship can then be quantified by plotting $[Ca^{2+}]$ at 50% myocyte relaxation (Mellor et al., 2012). Myocytes isolated from mice in estrus had higher levels of Ca^{2+} present at 50% contraction decay, which indicates that higher levels of

intracellular Ca^{2+} were required to interact with the myofilaments and activate contraction in cells from mice in estrus.

In order to obtain a more accurate measurement of myofilament Ca^{2+} sensitivity, actomyosin MgATPase activity was quantified. Actomyosin MgATPase activity is integral for cardiomyocyte contraction and provides quantification of the amount ATP that is hydrolyzed when myosin binds to actin to initiate cross-bridge formation (Venema and Kuo, 1993). Actomyosin MgATPase activity has previously been measured in hearts from OVX mice (Schaible et al., 1984; Wattanapermpool, 1998). However, the results of these studies are controversial, as both an increase and decrease in activity have been observed (Schaible et al., 1984; Wattanapermpool, 1998). The present study clearly showed that maximal actomyosin MgATPase activity did not vary with the estrous stages. Therefore, the highest [Ca^{2+}] generated similar levels of actomyosin MgATPase activity at all stages of the estrous cycle. However, this [Ca^{2+}] is supra-physiological and is not typical for functional myocardium. Interestingly, in the present study, the EC_{50} values for Ca^{2+} were significantly higher in estrus when compared to the three other stages of the estrous cycle. Additionally, a clear right-ward shift of the curve was also observed in the hearts isolated from mice in estrus. The results of the present study suggest that the myofilaments from mice in estrus were the least sensitive to Ca^{2+}.

Myosin plays a key role in cardiomyocyte contraction; it hydrolyzes ATP in order to provide the necessary energy for the cell to contract (Bers, 2002). The myosin heavy chain is an integral component for ATP hydrolysis, as myosin interacts with actin. There are two mammalian cardiac myosin isoforms, MHC-α

and MHC-β (Rayment et al., 1993). These isoforms are capable of complexing to form homo- or heterodimers. Cardiomyocytes with more MHC-α are capable of much faster contractions than those with more MHC-β (Palmiter et al., 1999). Myocytes isolated from mice in estrus had significantly faster contraction velocities compared to myocytes from the other stages of the estrous cycle. As no differences were seen in the velocities of the Ca^{2+} transient throughout the estrous cycle, another mechanism must underlie this finding. A potential explanation is that there may be a shift in the myosin isoforms that mediate contraction for mice in estrus. Therefore, it is possible that myocytes isolated from mice in estrus exhibit a shift toward the faster MHC-α inform, as the velocity of the contraction was significantly faster in estrus compared to the other estrous stages. Additional experiments would be required to test this hypothesis.

V. Contractile Responses and Ca^{2+} Concentrations in the Presence of β-adrenergic Stimulation in Cardiomyocytes From Females in the Four Stages of the Estrous Cycle.

The results of this study showed that fractional shortening and Ca^{2+} transient amplitudes remained significantly larger in myocytes isolated from mice in estrus in the presence of the β-AR agonist**,** ISO. By contrast, the EC_{50} values for fractional shortening and Ca^{2+} transient amplitude were not affected by the estrous cycle. These data show that maximal responses to ISO were largest in estrus, even though myocytes from all stages of the estrous cycle were equally sensitive to ISO.

A previous study has shown that β_1-AR are up-regulated in the hearts of OVX rats (Thawornkaiwong et al., 2003). Moreover, treatment with estrogen returns

receptor expression back to levels comparable to the sham-operated controls (Kam et al., 2004). This observation suggests that low estrogen states increase β_1-AR density. Therefore, the increased fractional shortening and Ca^{2+} transient amplitudes observed in estrus may be due to increased β_1-AR expression.

Up-regulation of the β_1-AR is also associated with enhanced PKA activity in OVX rats where estrogen levels are low (Kravtsov et al., 2007). As described previously, PKA is responsible for phosphorylating various components of the EC coupling pathway (Bers, 2002). Increases in peak $I_{Ca,L}$, RyR activity, and SERCA activity caused by PKA phosphorylation (Bers, 2002) could all contribute to the increased Ca^{2+} transient amplitudes seen in myocytes isolated from mice in estrus when compared to the other estrous stages. Thus, these results suggest that fractional shortening and Ca^{2+} transients are largest in myocytes isolated from mice in estrus in the presence of ISO due to an up-regulation of the β_1-AR and enhanced PKA activity.

VI. Contractile Responses and Ca^{2+} Concentrations following Acute Application of 17β-estradiol or the GPER agonist, G-1, in Cardiomyocytes From Females in the Four Stages of the Estrous Cycle.

i. Acute 17β-estradiol Application

The results of this study showed that acute application of 17β-estradiol had no effect on contractions or Ca^{2+} transients in myocytes isolated from mice in the four stages of the estrous cycle. Neither a physiological (100 pM) nor a supra-physiological (100 nM) concentration of 17β-estradiol altered cardiac contractile function. These findings contrast with the results of several studies investigating

the effects of acute application of 17β-estradiol on isolated cardiomyocytes. Jiang et al. were the first to show that acute application of a supra-physiological concentration of 17β-estradiol (30 μM) decreased contraction size, APD, and $I_{Ca,L}$ in ventricular myocytes isolated from male guinea-pigs (Jiang et al., 1992). Moreover, in a study in isolated human ventricular myocytes, a negative inotropic effect was observed within 5 mins following application of a supra-physiological concentration of 17β-estradiol (30 μM) (Sitzler et al., 1996). A decrease in $I_{Ca,L}$ has also been observed in rat ventricular myocytes following acute application of 30 μM of 17β-estradiol (Berger et al., 1997). The discrepancies between the results of the present study and these previous studies are likely due to the differences in the concentrations of estradiol used. The present study acutely administered supra-physiological concentrations that were 300 fold smaller than those administered in the previously mentioned studies. Taken together, the results of the present study suggest that acute application of physiological concentrations of 17β-estradiol have no effect on contractile function in myocytes regardless of the stage of the estrous cycle. Additionally, supra-physiological concentrations of approximately 30 μM 17β-estradiol appear to be required to modify contractile function, as even 100 nM estradiol had no effect on either contractions or Ca^{2+} transients at any estrous stage.

ii. Acute G-1 Application

The findings of this study showed that acute application of G-1 had no effect on contractions or Ca^{2+} transients in myocytes regardless of the estrous stage. No differences were observed in fractional shortening or Ca^{2+} transient amplitudes in

the presence of either 100 pM or 100 nM G-1. These findings are novel, as the impact of G-1 on cardiomyocyte function has not been investigated previously. The first study to investigate the effects of acute administration of G-1 on the heart examined G-1 in a model of ischemia-reperfusion injury in Langendorff hearts (Deschamps and Murphy, 2009). Deschamps and Murphy showed that pre-treatment for 10 mins with G-1 had no effect on contractile function. However, it did increase functional recovery and reduce infarct size following ischemia-reperfusion injury (2009). Therefore, pre-treatment with G-1 maintained cardiac contractile function in hearts following ischemic injury. Taken together, these results suggest that acute G-1 application does not affect myocyte contractile function in the absence of ischemia-reperfusion injury.

VII. Clinical Implications of Estradiol and Cardiovascular Function

i. The Menstrual Cycle and Cardiovascular Function

Serum estradiol levels fluctuate during the menstrual cycle from levels as low as 50 pg/ml during menstruation to approximately 850 pg/ml during the pre-ovulatory estradiol peak (Katzung, 2012). As previously mentioned, low levels of estradiol present during the luteal phase of the menstrual cycle are associated with the development of paroxysmal supraventricular tachycardia (Rosano et al., 1996). However, the mechanisms that underlie this developed tachycardia are poorly understood. The results of the present study indicate that intracellular Ca^{2+} levels are increased during estrus, which is the estrous stage that is characterized by the lowest estradiol levels (Saito et al., 2009). Thus, the results of the present study

suggest that increased intracellular Ca^{2+} levels may contribute to enhanced susceptibility to cardiac arrhythmias during phases of the menstrual cycle when estrogen levels are low.

ii. Contraception and Cardiovascular Function

Oral contraceptive pills (OCPs) are the most commonly prescribed method used to prevent pregnancy (Margolis et al., 2007). Approximately 80% of women have used OCPs within their lifetime. OCPs were developed to mimic the length of the typical menstrual cycle, with 28 days of fixed-dose estrogen and progesterone followed by 7 days of placebo to induce menstruation (Shufelt and Bairey Merz, 2009). Current OCPs are made up of a synthetic estrogen, known as ethinyl estradiol, and a gonane progestin, which have minimal androgenic effects (Shufelt and Bairey Merz, 2009). While a variety of studies have been conducted to investigate the effects of OCPs on cardiovascular risk factors, such as lipoprotein profiles (Barkfeldt et al., 2001; Endrikat et al., 2002), blood pressure (Cardoso et al., 1997; Chasan-Taber et al., 1996), and thrombosis (Jick et al., 1995; Sidney et al., 2004), no studies have directly studied cardiac function in pre-menopausal women taking OCPs. Serum estradiol levels are maintained at approximately 50 pg/ml in females taking OCPs (Gomella, 2007). This value closely approximates the lowest serum concentration of estradiol seen throughout the menstrual cycle (Katzung, 2012). Therefore, these results suggest that females taking OCPs have lower serum estradiol levels than other pre-menopausal women. The results of the present study showed that myocytes isolated from mice in estrus had the largest Ca^{2+} transient

amplitudes and increased fractional shortening. The observed Ca^{2+} loading is consistent with the increased prevalence of tachycardias seen in women during the luteal phase of their menstrual cycle. Taken together, these results suggest that females taking OCPs may have higher intracellular Ca^{2+} levels, which could increase their susceptibility to various heart diseases, such as cardiac arrhythmias.

VIII. Limitations

The present study investigated the effects of the estrous cycle on contractile function and Ca^{2+} concentrations in isolated mouse ventricular myocytes. While studies on individual cardiomyocytes are integral in understanding how contractions and Ca^{2+} transients are altered at the level of the single cell, it may not be entirely possible to directly translate these findings on to the whole heart. A logical progression would be to assess cardiac contractile function in whole hearts isolated from mice in the four stages of the estrous cycle. The Langendorff heart preparation, which involves retrograde perfusion of the heart through the aorta, allows the heart to be studied without the influence of other organ systems (Bell et al., 2011). Myocardial contractility and left ventricular function can be easily assessed through insertion of a left ventricular balloon(Bell et al., 2011). The advantage of this preparation is the fact that the myocardium is intact and the cardiomyocytes continue to interact to generate the synchronized contraction of the heart. The myocytes are subjected to hemodynamic load and are exposed to the stress and strain associated with being interconnected, which is an integral part of cardiac function that is lost in single cell studies.

In addition, functional experiments could be conducted *in vivo* in order to assess the effect of the estrous cycle on the heart and how these changes influence the organism as a whole. For example, an echocardiogram using continuous wave Doppler ultrasound could be used as a non-invasive tool to assess any structural or functional differences in the heart at the various estrous stages (Kayrak et al., 2011). Whole heart and *in vivo* studies would serve to provide a complete picture as to how the estrous cycle influences overall heart function.

Finally, the majority of this thesis focused on the effects of the sex steroid hormone, estrogen, and its effects on the myocardium. While it is established that estrogen modulates cardiovascular function, the potential effects of the other circulating sex hormones cannot be neglected. The effects of progesterone, FSH, and LH on the heart are poorly understood. Further studies are required to determine if these hormones are able to influence heart function.

IX. Summary

The present study demonstrates that group-housed female mice have irregular estrous cycles, however, they can be induced to cycle through exposure to bedding collected from cages housing male mice. Fractional shortening and Ca^{2+} transient amplitudes are significantly larger in myocytes isolated from mice in estrus when compared to the other estrous stages. The effects of the estrous cycle on myocyte contractile function are also maintained when cells are stimulated at a physiological pacing rate or when they are subjected to β-adrenergic stimulation. Additionally, acute application of 17β-estradiol or the GPER agonist, G-1, did not

affect contractile function or Ca^{2+} concentrations in myocytes isolated throughout the estrous cycle. This is the first study to demonstrate that myocyte contractile function is altered through fluctuations in sex steroid levels throughout the estrous cycle. The results of this study have important implications, since alterations in Ca^{2+} homeostasis are associated with arrhythmia generation and various cardiovascular diseases. These findings also suggest that susceptibility to an adverse cardiovascular event may vary with cyclical changes in estrogen levels.

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