METABOLIC REGULATION OF FAST- AND SLOW-TWITCH SKELETAL MUSCLES BY ESTRADIOL AND PROGESTERONE IN FEMALES AND THEIR IMPACT DURING ISCHEMIA

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Physiology
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Metabolic regulation of fast- and slow-twitch skeletal muscles by estradiol and progesterone in females and their impact during ischemia

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Abstract

The roles of estradiol and progesterone were investigated to determine the effect of these hormones on metabolism in skeletal muscle composed of different fiber types (slow-twitch, fast-twitch and their various sub-types) which have different metabolic properties. The metabolic implications of these sex hormones were also tested under ischemic conditions. Female Sprague-Dawley rats were ovariectomized and given one of the following for 120 days: estradiol (ET), intermittent-estradiol (IE), progesterone (P), both IE and P (IE+P), estrogen receptor (ER) antagonist ICI 182,780 (ICI), selective ER modulator Raloxifene (RLX). Key statistically significant findings included that estradiol decreased glycogen phosphorylase and phosphofructokinase activity in fast-twitch muscles with greater total fast-twitch fiber content. Progesterone decreased in vivo glycogen by 25% in slow-twitch muscles. This response was only found in fast-twitch muscles when IE+P was given. During ischemia, IE+P reduced glycogen consumption irrespective of muscle type, which was attributed to lower in vivo glycogen levels. In vivo creatine phosphate (CP) levels in females given IE+P were reduced by 25%, irrespective of muscle type. Ischemic CP consumption was decreased only in fast-twitch muscles of IE+P females who had lower in vivo CP levels. ER studies established that ICI significantly lowered in
in vivo CP levels by 70-80% in both fast- and slow-twitch muscles, resulting in reduced ischemic CP consumption. RLX did not appear to modulate metabolism in these studies. These results demonstrated that the principle female sex hormones are important regulators of glycogen and CP metabolism and that the ER blockade decreases CP content in skeletal muscle. Moreover, ovarian steroid hormone regulation of glycogen metabolism is muscle fiber specific, whereas that of CP metabolism is not muscle fiber specific.
Acknowledgments

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TABLE OF CONTENTS

Abstract ........................................ ii
Acknowledgements ................................ iv
Table of Contents .................................v
List of Abbreviations ......................... xii
List of Figures ................................ xvii
List of Tables ................................... xix
List of Appendices ............................. xxi

CHAPTER 1 ................................................ 1

1.0 GENERAL INTRODUCTION .................. 2

1.1 Skeletal Muscle Properties ............... 3
   1.1.a General Anatomy of Skeletal Muscle 3
   1.1.b Muscle Fiber Types ................... 5
   1.1.c Slow-Twitch Fibers (Type I) ........ 5
   1.1.d Fast-Twitch Fibers (Type II) ....... 5
   1.1.e Significance of Molecular Diversity of Myofibrillar Proteins 6
   1.1.f Effect of Sex Hormones on Skeletal Muscle Fiber Types 7
   1.1.g Metabolic Differences Between Fiber Types 8
   1.1.h Skeletal Muscle Ischemia – Changing the Flow of Things 12

1.2 Overview of Estradiol & Progesterone on Skeletal Muscle 16
   1.2.a What is Known in Postmenopausal Women 16
   1.2.b Sex-Related Differences in Baseline Skeletal Muscle Metabolism 17
   1.2.c Sex Differences on Substrate Utilization during Exercise 19
   1.2.d Ovarian Cycle & Exercise Metabolism 20
   1.2.e Estradiol & Carbohydrate Metabolism during Exercise 21
   1.2.f Progesterone & Carbohydrate Metabolism during Exercise 22
   1.2.g Female Sex Hormones on Skeletal Muscle Enzyme Activity 23

1.3 The Ovarian Hormones Estradiol & Progesterone 25
   1.3.a Ovarian Hormones: Biosynthesis 25
   1.3.b Overview of Steroid Hormone Receptors 29
   1.3.c ER Structure and Function .......... 29
   1.3.d ER Isoforms .......................... 31
   1.3.e Co-activators of the ER ............. 32
   1.3.f Co-repressors of the ER ............ 33
   1.3.g Non-genomic Mechanisms of the ER 34
   1.3.h ER Isoform Expression in Skeletal Muscle 34
   1.3.i ER Antagonist ICI 182,780 ........... 35
1.3.j Selective ER Modulator (SERM) Raloxifene 36
1.3.k 1.3.k. ER Binding Properties 36

1.4 Rationale 38
1.5 Hypotheses 41

CHAPTER 2 MATERIALS & METHODS 42

2.1 Animals 43

2.2 Hormone Manipulation Models 43

2.2.a Study 1 43
2.2.b Study 2 45
2.2.c Study 3 47

2.3 Final Acute Experimental Model 48

2.3.a Surgical Preparation 48
2.3.b Skeletal Muscle Biopsies 49
2.3.c Skeletal Muscle Ischemia 51

2.4 Biochemical Analyses 51

2.4.a Enzymatic Analyses 51
2.4.b Glycogen, Lactate, ATP, CP Analyses 57
2.4.c Plasma 17β-Estradiol Analyses 57

2.5 Mathematical & Statistical Analyses 58

2.5.a Definition of Net Change in Metabolic Variables 58
2.5.b Definition of Net Change in Metabolic Variables Expressed as a Percentage of In Vivo Values 59
2.5.c Study 1 59
2.5.d Study 2 60
2.5.e Study 3 61

CHAPTER 3 RESULTS 63

3.1 Study 1: Regulation of metabolic enzymes by estradiol in fast-twitch skeletal muscles 64

3.1.a Creatine Kinase (CK) 64
3.1.a.(i) The effect of estradiol on CK activity in the GRAC, SMT, EDL muscles 64
3.1.a.(ii) The effect of differing fast-twitch muscles on CK 64
activity in each experimental group

3.1.b Phosphofructokinase (PFK)
3.1.b.(i) The effect of estradiol on PFK activity in the GRAC, SMT, EDL muscles
3.1.b.(ii) The effect of differing fast-twitch muscles on PFK activity in each experimental group

66

3.1.c Glycogen Phosphorylase (GP)
3.1.c.(i) The effect of estradiol on GP activity in the GRAC, SMT, EDL muscles
3.1.c.(ii) The effect of fast-twitch muscles on GP activity in each experimental group

68

3.1.d Lactate Dehydrogenase (LDH)
3.1.d.(i) The effect of estradiol on LDH activity in the GRAC, SMT, EDL muscles
3.1.d.(ii) The effect of fast-twitch muscles on LDH activity in each experimental group

70

3.1.e Citrate Synthase (CS)
3.1.e.(i) The effect of estradiol on CS activity in the GRAC, SMT, EDL muscles
3.1.e.(ii) The effect of fast-twitch muscles on CS activity in each experimental group

72

3.1.f Succinate Dehydrogenase (SDH)
3.1.f.(i) The effect of estradiol on SDH activity in the GRAC, SMT, EDL muscles
3.1.f.(ii) The effect of fast-twitch muscles on SDH activity in each experimental group

72

3.1.g Beta Hydroxyacyl Dehydrogenase (βHAD)
3.1.g.(i) The effect of estradiol on βHAD activity in the GRAC, SMT, EDL muscles
3.1.g.(ii) The effect of fast-twitch muscles on βHAD activity in each experimental group

73

3.1.h Cytochrome C Oxidase (COX)
3.1.h.(i) The effect of estradiol on COX activity in the GRAC, SMT, EDL muscles
3.1.h.(ii) The effect of fast-twitch muscles on COX activity in each experimental group

74

Summary of Results for Study 1

75
3.2 Study 2: The role of intermittent-estradiol and progesterone in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

3.2.a  In vivo CP
3.2.a.(i) The effect of female sex hormones on in vivo CP levels in fast- and slow-twitch muscles
3.2.a.(ii) The effect of fast- and slow-twitch muscles on in vivo CP levels within each experimental group

3.2.b  In vivo Glycogen
3.2.b.(i) The effect of female sex hormones on in vivo glycogen levels in fast- and slow-twitch muscles
3.2.b.(ii) The effect of fast- and slow-twitch muscles on in vivo glycogen levels within each experimental group

3.2.c  In vivo ATP
3.2.c.(i) The effect of female sex hormones on in vivo ATP levels in fast- and slow-twitch muscles
3.2.c.(ii) The effect of fast- and slow-twitch muscles on in vivo ATP levels within each experimental group

3.2.d  In vivo Lactate
3.2.d.(i) The effect of female sex hormones on in vivo lactate levels in fast- and slow-twitch muscles
3.2.d.(ii) The effect of fast- and slow-twitch muscles on in vivo lactate levels within each experimental group

3.2.e  Ischemic CP
3.2.e.(i) The effect of female sex hormones on ischemic CP levels in fast- and slow-twitch muscles
3.2.e.(ii) The effect of fast- and slow-twitch muscles on ischemic CP levels within each experimental group

3.2.f  Ischemic Glycogen
3.2.f.(i) The effect of female sex hormones on ischemic glycogen levels in fast- and slow-twitch muscles
3.2.f.(ii) The effect of fast- and slow-twitch muscles on ischemic glycogen levels within each experimental group

3.2.g  Ischemic ATP
3.2.g.(i) The effect of female sex hormones on ischemic ATP levels in fast- and slow-twitch muscles
3.2.g.(ii) The effect of fast- and slow-twitch muscles on ischemic ATP levels within each experimental group
ischemic ATP levels within each experimental group

3.2.h Ischemic Lactate
3.2.h.(i) The effect of female sex hormones on ischemic lactate levels in fast- and slow-twitch muscles
3.2.h.(ii) The effect of fast- and slow-twitch muscles on ischemic lactate levels within each treatment group

Summary of Results for Study 2

3.3 Study 3: The role of ER in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

3.3.a In vivo CP
3.3.a.(i) The role of skeletal muscle ER on in vivo CP levels in the fast-twitch EDL and slow-twitch SOL muscles
3.3.a.(ii) The effect of fast- and slow-twitch muscles on in vivo CP levels within each treatment group

3.3.b In vivo Glycogen
3.3.b.(i) The role of skeletal muscle ER on in vivo glycogen levels in the fast-twitch EDL and slow-twitch SOL muscles
3.3.b.(ii) The effect of fast- and slow-twitch muscles on in vivo glycogen levels within each treatment group

3.3.c In vivo ATP
3.3.c.(i) The role of skeletal muscle ER on in vivo ATP levels in the fast-twitch EDL and slow-twitch SOL muscles
3.3.c.(ii) The effect of fast- and slow-twitch muscles on in vivo ATP levels within each treatment group

3.3.d In vivo Lactate
3.3.d.(i) The role of skeletal muscle ER on in vivo lactate levels in the fast-twitch EDL and slow-twitch SOL muscles
3.3.d.(ii) The effect of fast- and slow-twitch muscles on in vivo lactate levels within each treatment group

3.3.e Ischemic CP
3.3.e.(i) The role of skeletal muscle ER on ischemic CP levels in fast- and slow-twitch muscles
3.3.e.(ii) The effect of fast- and slow-twitch muscles on ischemic CP levels within each treatment group
CHAPTER 4

4.0 Discussion

4.1 Study 1: Regulation of metabolic enzymes by estradiol in fast-twitch skeletal muscles

4.1.a Glycogen Phosphorylase (GP)
4.1.b Phosphofructokinase (PFK)
4.1.c Role Transcription Factor Nur77 May Have in Regulating GP and PFK activity
4.1.d Lactate Dehydrogenase (LDH)
4.1.e Creatine Kinase (CK)
4.1.f Citrate Synthase (CS), Succinate Dehydrogenase (SDH), beta-hydroxyacyl CoA dehydrogenase (βHAD), and Cytochrome C Oxidase (COX)

Summary of Discussion for Study 1

4.2 Study 2: The role of intermittent-estradiol and progesterone in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

4.2.a In vivo effects of intermittent estradiol and progesterone
4.2.b Effect of intermittent estradiol and progesterone on ischemic muscle metabolism

Summary of Discussion for Study 2

4.3 Study 3: The role of ER in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

4.3.a In vivo effects of ICI and RLX
4.3.b The effects of ICI and RLX on ischemic muscle metabolism

4.4 General Discussion

4.5 Conclusions

4.6 Limitations

4.7 Future Directions

5.0 References

APPENDICES

1 Muscle Fiber Distribution of Various Skeletal Muscles Referenced in Thesis
2 Uterus to Body Weight Ratios of Experimental Animals of Studies 1, 2, 3
3 Plasma 17β-Estradiol Levels in Experimental Animals of Studies 1, 2, 3

COPYRIGHT ACKNOWLEDGEMENTS
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AF-1</td>
<td>activation function-1</td>
</tr>
<tr>
<td>AF-2</td>
<td>activation function-2</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>1,3-BPG</td>
<td>1,3-bisphosphoglycerate</td>
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<tr>
<td>βHAD</td>
<td>beta hydroxyacyl dehydrogenase</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>bw</td>
<td>body weight</td>
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<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
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<tr>
<td>CBG</td>
<td>corticosteroid-binding protein</td>
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<td>CBP</td>
<td>p300 cAMP response element binding protein</td>
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<tr>
<td>CK</td>
<td>creatine kinase</td>
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<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>COX</td>
<td>cytochrome C oxidase</td>
</tr>
<tr>
<td>CP</td>
<td>creatine phosphate</td>
</tr>
<tr>
<td>CS</td>
<td>citrate synthase</td>
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<td>d</td>
<td>day</td>
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<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dw</td>
<td>dry weight</td>
</tr>
<tr>
<td>E</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>estradiol</td>
</tr>
<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERα</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>estradiol treated</td>
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**F**

F  
female

F-6-P  
fructose-6-phosphate

F-1,6-BP  
fructose-1,6-bisphosphate

FP  
follicular phase

FSH  
follicular stimulating hormone

FT  
fast-twitch

**G**

g  
grains

G-1-P  
glucose-1-phosphate

G-6-P  
glucose-6-phosphate

GLUT  
glucose transporter

Gly-3-P  
glyceraldehyde-3-phosphate

GP  
glycogen phosphorylase

GRAC  
gracilis

GRIP-1  
gluocorticoid receptor interacting protein

GS  
glycogen synthase

**H**

H⁺  
hydrogen ion

HADH  
β-hydroxyacyl-coenzyme A dehydrogenase

HAT  
histone acetyltransferase activity

3β-HD  
3 beta hydroxysteroid dehydrogenase

HCO₃⁻  
bicarbonate ion

HCl  
hydrogen chloride

HK  
hexokinase

H₂O  
water

hr  
hour

HRE  
hormone response element

hsp  
heat shock protein

**I**

ICl  
ICI 182,780

IE  
intermittent estrogen

INT  
intact

**K**

K⁺  
potassium ion

KCl  
potassium chloride

g  
kilogram
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<tr>
<td>KHCO₃</td>
<td>potassium bicarbonate</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>L</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LP</td>
<td>luteal phase</td>
</tr>
<tr>
<td>M</td>
<td>molarity</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
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<td>M-CK</td>
<td>muscle creatine kinase</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
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<td>MgCl₂</td>
<td>magnesium chloride</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>Mi-CK</td>
<td>mitochondrial creatine kinase</td>
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<td>min</td>
<td>minutes</td>
</tr>
<tr>
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<td>milliliter</td>
</tr>
<tr>
<td>mmHg</td>
<td>millimeters of mercury</td>
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<td>messenger ribonucleic acid</td>
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<td>myosin light chain</td>
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<td>sodium chloride</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
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<td>NCOR</td>
<td>nuclear receptor corepressor</td>
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<td>ng</td>
<td>nanogram</td>
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<td>nm</td>
<td>nanometer</td>
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<td>NS</td>
<td>not significant</td>
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<td>O₂</td>
<td>oxygen</td>
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<td>P</td>
<td>progesterone</td>
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<td>perchloric acid</td>
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PFK phosphofructokinase
pg picogram
$P_i$ inorganic phosphate
PK pyruvate kinase
PL plantaris

R
RBA relative binding affinity
RER respiratory exchange ratio
RG red gastrocnemius
RLX Raloxifene

S
SCC side-chain cleavage enzyme complex
SD standard deviation
SDH succinate dehydrogenase
SERM selective estrogen receptor modulator
SHAM sham-operated
SHBG sex hormone-binding globulin
SMT semitendinosus
SMRT silencing mediator for retinoid and thyroid receptor
SOL soleus
SRC steroid receptor co-activator
ST slow-twitch

T
TAN total adenine nucleotides
Tm tropomyosin
Tn troponin
TnC troponin C
TnI troponin I
TnT troponin T

U
µl microlitre
µM micromolar
uw uterus weight

V
VL vastus lateralis
VLP vastus lateralis profundus
VLS  vastus lateralis superficialis
\( \dot{V}O_{2\text{max}} \)  maximum oxygen consumption
\( V_{\text{max}} \)  maximum velocity
\( \text{vs} \)  versus

\( W \)

\( \text{WG} \)  white gastrocnemius
\( \text{ww} \)  wet weight
\( \text{wt} \)  weight

\( Z \)

\( \text{ZnCl}_2 \)  zinc chloride

**LIST OF SYMBOLS**

\( \alpha \)  alpha
\( \sim \)  approximately
\( \beta \)  beta
\( \Delta \)  delta
\( ^\circ \text{C} \)  degrees Celsius
\( = \)  equals
\( \leq \)  less than or equal to
\( \mu \)  micro
\( - \)  negative
\( \% \)  percent
\( \pm \)  plus or minus
\( \times \)  multiply by
# LIST OF FIGURES

## CHAPTER 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The structural organization of skeletal muscle from the gross to the molecular level</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Metabolic pathways active during global ischemia in skeletal muscle</td>
<td>14</td>
</tr>
<tr>
<td>1.3</td>
<td>The synthesis of estradiol and progesterone from cholesterol</td>
<td>27</td>
</tr>
<tr>
<td>1.4</td>
<td>LH and FSH regulation of estradiol and progesterone synthesis in the thecal and granulosa cells of the ovary</td>
<td>28</td>
</tr>
<tr>
<td>1.5</td>
<td>Schematic structure of the two ER isoforms and the percent homology between them</td>
<td>31</td>
</tr>
</tbody>
</table>

## CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.a</td>
<td>Timeline for pellet protocol for female rats in Studies 1, 2, and 3</td>
<td>44</td>
</tr>
<tr>
<td>2.1</td>
<td>Animal groups used in Study 1</td>
<td>44</td>
</tr>
<tr>
<td>2.2</td>
<td>Animal groups used in Study 2</td>
<td>45</td>
</tr>
<tr>
<td>2.3</td>
<td>Plasma estradiol levels in four female Sprague-Dawley rats illustrating dosing regimen of IE pellets.</td>
<td>46</td>
</tr>
<tr>
<td>2.4</td>
<td>Animal groups used in Study 3</td>
<td>47</td>
</tr>
<tr>
<td>2.5</td>
<td>Muscles and metabolic variables measured in Study 1</td>
<td>49</td>
</tr>
<tr>
<td>2.6</td>
<td>Muscles and metabolic variables measured in Studies 2 &amp; 3</td>
<td>50</td>
</tr>
<tr>
<td>2.7.a</td>
<td>Metabolic reactions for creatine kinase analyses</td>
<td>52</td>
</tr>
<tr>
<td>2.7.b</td>
<td>Metabolic reactions for phosphofructokinase analyses</td>
<td>53</td>
</tr>
<tr>
<td>2.7.c</td>
<td>Metabolic reactions for glycogen phosphorylase analyses</td>
<td>54</td>
</tr>
<tr>
<td>2.7.d</td>
<td>Metabolic reaction for lactate dehydrogenase analyses</td>
<td>54</td>
</tr>
<tr>
<td>2.7.e</td>
<td>Metabolic reactions for citrate synthase analyses</td>
<td>55</td>
</tr>
</tbody>
</table>
CHAPTER 3

Figure 3.1.1 PFK activity of the three different fast-twitch muscles 67
Figure 3.1.2 GP activity of the three different fast-twitch muscles 69
Figure 3.1.3 LDH activity of the three different fast-twitch muscles 71
Figure 3.2.1 \textit{In vivo} CP levels 78
Figure 3.2.2 \textit{In vivo} glycogen levels 80
Figure 3.2.3 \textit{In vivo} ATP levels 82
Figure 3.2.4 (i) Net CP consumption (ii) % CP consumed during ischemia 86
Figure 3.2.5 Glycogen levels at 3 hours of ischemia 90
Figure 3.2.6 (i) Net glycogen consumption (ii) % glycogen consumed during ischemia 91
Figure 3.2.7 (i) Net ATP consumption (ii) % ATP consumed during ischemia 95
Figure 3.3.1 \textit{In vivo} CP levels 103
Figure 3.3.2 (i) Net CP consumption (ii) % CP consumption during ischemia 112

CHAPTER 4

Figure 4.1 Proposed potential mechanism illustrating how estradiol (E2) could reduce GP and PFK activity levels in fast-twitch muscles via Nur77 124
Figure 4.2 Summary of significant findings of Study 1 which examined 129
the effect of estradiol on key enzymes involved in energy production in the fast-twitch muscles gracilis, semitendinosus, and extensor digitorum longus muscles

Figure 4.3 Summary of significant findings of thesis work illustrating the effects of estradiol, P, and ICI on in vivo muscle metabolism

LIST OF TABLES

CHAPTER 1

Table 1.1 Myosin, actin, troponin (Tn), and tropomyosin (Tm) isoforms of adult fast and slow rat skeletal muscles 8

Table 1.2 Differences in oxidative and glycolytic potential among different types of skeletal muscles 10

Table 1.3 Summary of sex differences in the activity of different metabolic enzymes from the fast-twitch vastus lateralis muscles of men and women 18

Table 1.4 Relative binding affinities (RBA) of various ligands to different isoforms of the ER 37

CHAPTER 3

Table 3.1.1 Enzyme activity of CK, GP, PFK, LDH, CS, SDH, βHAD, and COX (expressed as moles/kg protein/hr) in the GRAC, SMT, and EDL muscles of female Sprague-Dawley rats who were either sham-operated (INT), ovariectomized (OVX), or estradiol treated (ET) 65

Table 3.2.1 CP content (µmol/g dw) from in vivo and ischemic skeletal muscles 87

Table 3.2.2 Glycogen content (µmol/g dw) from in vivo and ischemic tissue 92

Table 3.2.3 ATP content (µmol/g dw) from in vivo and ischemic tissue 96

Table 3.2.4 Lactate content (µmol/g dw) from in vivo and ischemic tissue 98

Table 3.3.1 CP content (µmol/g dw) from in vivo and ischemic skeletal muscles 106
<table>
<thead>
<tr>
<th>Table 3.3.2</th>
<th>Glycogen content (µmol/g dw) from <em>in vivo</em> and ischemic skeletal muscles</th>
<th>107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.3.3</td>
<td>ATP content (µmol/g dw) of <em>in vivo</em> and ischemic skeletal muscles</td>
<td>108</td>
</tr>
<tr>
<td>Table 3.3.4</td>
<td>Lactate content (µmol/g dw) of <em>in vivo</em> and ischemic skeletal muscles</td>
<td>109</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix 1</td>
<td>Muscle Fiber Distribution of Various Skeletal Muscles Referenced in Thesis</td>
<td>183</td>
</tr>
<tr>
<td>Appendix 2</td>
<td>Uterus to Body Weight Ratios of Experimental Animals of Studies 1, 2, 3</td>
<td>185</td>
</tr>
<tr>
<td>Appendix 3</td>
<td>Plasma 17β-Estradiol Levels in Experimental Animals of Studies 1, 2, 3</td>
<td>188</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.0 GENERAL INTRODUCTION

Over the decades, 17β-estradiol (estradiol) and progesterone have been shown to influence the metabolic profile of women during exercise (Isacco et al., 2012; Tarnopolsky, 2008; D'eon & Braun, 2002; Tarnopolsky & Saris 2001) which may explain the exercise-related sex differences in metabolism, such as a greater promotion of fat metabolism in women compared to men. Sex differences in exercise-related metabolism may be attributed to the effects estradiol and progesterone have on skeletal muscle glucose metabolism. The molecular mechanisms that these hormones utilize are unclear, but the presence of estrogen receptors (ER) in skeletal muscle suggests genomic control of metabolic pathways.

The work presented in this doctoral thesis focuses on the metabolic adaptations that the sex hormones estradiol and progesterone exert in different types of skeletal muscle and identify the role of the ER as a potential mechanism. Therefore, this thesis work provides new knowledge identifying the relationship between how these sex hormones influence the metabolism of skeletal muscle of various fiber types, the role of the ER in this response, and tests these factors under ischemic conditions.

The upcoming sections in the Introduction provide an overview of (1) skeletal muscle characteristics and metabolic differences in fiber types, (2) the effect of estradiol and progesterone in skeletal muscle, and (3) details regarding the ER in skeletal muscle, which will provide information needed to understand the rationale and hypotheses of this thesis.
1.1 SKELETAL MUSCLE PROPERTIES

Approximately 40% of the body is composed of skeletal muscle which is the main organ responsible for movement. Some of the diversity of skeletal muscle function is attributed to the variety of fiber types which have their own functional capacities matched with the appropriate metabolic machinery. Because of this diversity, it is important to investigate the different types of skeletal muscle, rather than assume that skeletal muscles are all the same. In this section, a general overview of skeletal muscle structure and function is provided. The relevant structures that contribute to the diversity of muscle fibers are discussed, followed by a review of their metabolic differences to appreciate the heterogeneity of skeletal muscle.

1.1.a General Anatomy of Skeletal Muscle

Skeletal muscle consists of numerous fibers (see Figure 1.1), which in turn are made of smaller subunits. As illustrated in Figure 1.1, a skeletal muscle fiber is a specialized type of cell that is multi-nucleated and contains several hundred to several thousand myofibrils. Each myofibril has about 1500 myosin filaments and 3000 actin filaments lying side by side which are two of the key proteins involved in muscle contraction. Because myosin and actin filaments partially interdigitate, myofibrils have alternating light and dark bands. The light bands contain only actin filaments and are called the I-band. The dark bands contain myosin filaments as well as the ends of actin filaments where they overlap the myosin and are called the A-band. The ends of actin filaments are attached to the Z-disc. From this disc, actin filaments extend in both directions to interdigitate with myosin filaments. Skeletal muscle's characteristic striated appearance is due to muscle fibers and individual myofibrils having light and dark bands. The portion of the myofibril that lies between two successive Z-discs is called the sarcomere. Skeletal muscle fibers have a tremendous volume of mitochondria that lie between and parallel to the
myofibrils in order to supply the myofibrils with a large amount of adenosine triphosphate (ATP) required during muscle contraction.

Figure 1.1. The structural organization of skeletal muscle from the gross to the molecular level. Reproduced with permission from Guyton and Hall, Textbook of Medical Physiology, pg 74, 1996.
1.1.b Muscle Fiber Types

There are 2 main skeletal muscle fiber types in the body, slow-twitch and fast-twitch fibers, which in the literature are also known as Type I and Type II fibers, respectively. These muscle fibers have individual metabolic adaptations to satisfy their functional needs.

1.1.c Slow-Twitch Fibers (Type I)

Slow-twitch fibers contract more slowly compared to fast-twitch fibers, produce less mechanical power, and are adapted for long, slow, posture-maintaining activity (Schiaffino & Reggiani, 2011). These are smaller fibers that have a more extensive blood vessel system and capillary network which is the reason why they are often referred to as red muscle. This vascularization provides the extra amounts of oxygen needed for oxidative metabolism, which is its primary metabolic pathway for energy production.

1.1.d Fast-Twitch Fibers (Type II)

There are several categories of fast-twitch fibers in mammals, identified as Type IIa, IIb, and IIx; however, the Type IIb isoform does not exist in humans (Sant'Ana Pereira et al., 1997). Fast-twitch fibers are much larger compared to slow-twitch fibers and are used for rapid, powerful muscle contractions (Schiaffino & Reggiani, 2011). Fast-twitch fibers tend to fatigue more quickly; hence, they are better adapted for intermittent and strong bursts of contractile activity. These fibers rely mainly on glycolytic pathways for energy production and have a less extensive blood supply. In some cases, muscles that have a high proportion of Type IIb fibers are referred to as white muscles (Table 2, Appendix 1).
1.1.e Significance of Molecular Diversity of Myofibrillar Proteins

The different skeletal muscle fiber types are made possible by the existence of myofibrillar protein isoforms (Schiaffino & Reggiani, 2011; Bottinelli & Reggiani, 2000). A brief overview of the different contractile proteins in the different fiber types will be reviewed to understand their functional and metabolic characteristics.

The myofibrillar proteins that constitute the thick and thin filaments of the sarcomere have been shown to exist as distinct isoforms. The thick filament is composed of myosin and several myosin-binding proteins. Myosin is a hexamer made of 2 myosin heavy chains (MHCs) and 2 pairs of myosin light chains (MyLCs) named essential MyLC1 and regulatory MyLC2. Each myosin is characterized by a ‘head’ that corresponds to the motor domain, and contains the ATP-binding and actin-binding sites and a long ‘tail’ composed of MHC (Schiaffino & Reggiani, 2011). This connection is held together by a thin neck, to which the essential and regulatory MyLCs are bound. In adult skeletal muscle of different mammalian species, there are 4 predominant MHC isoforms: MHC-1(β/slow), MHC-IIa, MHC-IIx, and MHC-IIb. There are also different isoforms of MLC in fast-twitch and slow-twitch fibers that are summarized in Table 1.1. These proteins collectively contribute to both the structural differences and the functional properties of these muscles. The maximum velocity ($V_{\text{max}}$) and peak power of myosin ranges from lowest in Type I fibers to greatest in Type IIb fibers in the following order: I < IIa < IIx < IIb (Schiaffino & Reggiani, 2011). Also, muscles and muscle fibers containing fast MHC isoforms consume ATP at a faster rate than those containing slow isoforms (Rivero et al., 1999). The ATP splitting rate, or rate of ATP consumption, in the rat fast-twitch extensor digitorum longus (EDL) muscle is approximately 6 times greater than the rat slow-twitch soleus (SOL) muscle (0.8-1.15 ATP hydrolyzed/myosin head/s vs 0.22 ATP hydrolyzed/myosin head/s, respectively) (Schiaffino & Reggiani, 1996). The ATP splitting rate also differs among the
different fast-twitch fibers such that Type IIb fibers have the highest activity (1.15 ATP hydrolyzed/myosin head/s) and Type IIa fibers (0.82 ATP hydrolyzed/myosin head/s) have lower values (Schiaffino & Reggiani, 1996). In humans, ATP consumption ranges from lowest in Type I fibers to greatest in Type IIb fibers in the following order: I < IIa < IIx (Schiaffino & Reggiani, 2011). In rats, myosin ATPase activity shows a similar pattern and also ranges from the lowest in Type I to greatest in Type IIb fibers in the following order: I < IIa < II\textsubscript{d/x} < IIb (Rivero et al., 1999).

The major components of the thin filament are actin, tropomyosin, and the troponin complex [troponin C (TnC), troponin T (TnT), troponin I (TnI)] (Schiaffino & Reggiani, 1996). There are also different isoforms of these proteins in fast and slow fibers (summarized in Table 1.1) that are responsible for contributing to the range of functional properties observed in muscle fibers (Schiaffino & Reggiani, 2011).

1.1.f Effect of Sex Hormones on Skeletal Muscle Fiber Types

Studies have investigated the role of estradiol on MHC isoforms, but that of progesterone has not been explored. Specifically, in the fast-twitch plantaris muscle, the percentages MHCI and MHCIIa fibers were not altered by ovariectomy (OVX), but the percentage of MHCIIx fibers was reduced with OVX and returned to intact female values with estrogen treatment (Piccone et al., 2004). However, in another study, in the slow-twitch SOL muscle, neither MHCI, IIa or IIx fiber proportions were affected by OVX or estrogen treatment (McCormick et al., 2004). Thus, myosin isoforms in slow-twitch muscles are not affected by female sex hormones, whereas in fast-twitch muscles, only the MHCIIx isoform was regulated by estradiol and thereby have the potential to impact on metabolic properties.
Table 1. Myosin, actin, troponin (Tn), and tropomyosin (Tm) isoforms of adult fast- and slow-twitch rat skeletal muscles. Data taken from Schiaffino & Reggiani, 1996.

### 1.1.g Metabolic Differences Between Fiber Types

Functional differences in skeletal muscle fibers are associated with metabolic differences to satisfy energy requirements. Generally, fast-twitch fibers rely primarily on glycolytic pathways to generate energy (ATP) to match the rapid muscular contractions. In contrast, slow-twitch muscles primarily generate ATP via oxidative pathways which is better suited for longer sustained contractile function (Schiaffino & Reggiani, 2011). There are several metabolic
pathways for skeletal muscles to produce energy, and these pathways are present in each fiber type. Fiber type differences exists in the availability of substrate and the enzymatic potential of key regulatory enzymes of these metabolic pathways. Key enzymes in skeletal muscle that contribute to glycolytic and oxidative sources of energy are listed below:

<table>
<thead>
<tr>
<th>ENZYME:</th>
<th>ENERGY SOURCE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>creatine kinase (CK)</td>
<td>creatine phosphate shuttle</td>
</tr>
<tr>
<td>phosphofructokinase (PFK)</td>
<td>glycolytic potential</td>
</tr>
<tr>
<td>glycogen phosphorylase (GP)</td>
<td>glycogen metabolism</td>
</tr>
<tr>
<td>lactate dehydrogenase (LDH)</td>
<td>anaerobic potential</td>
</tr>
<tr>
<td>citrate synthase (CS) and succinate dehydrogenase (SDH)</td>
<td>oxidative potential</td>
</tr>
<tr>
<td>β-hydroxyacyl-coenzyme A dehydrogenase (βHAD)</td>
<td>lipid metabolism</td>
</tr>
<tr>
<td>Cytochrome C Oxidase (COX)</td>
<td>electron transport chain</td>
</tr>
</tbody>
</table>

Many papers have documented the enzymatic differences between fast- and slow-twitch fibers, some of which are summarized in Table 1.2. The activities of PFK, LDH and CK are greater in Type II fibers compared to Type I fibers, whereas the activities of CS, SDH and βHAD are greater in Type I compared to Type II fibers (Table 1.2). In Type IIa muscles, such as the red gastrocnemius (RG) or vastus lateralis profundus (VLP) muscles, not only do they have a relatively high oxidative potential, but they also exhibit a relatively high glycolytic potential compared to the Type I muscles. As illustrated in Table 1.2, there is also a range in the activity of enzymes in slow- versus fast-twitch muscles and even among various fast-twitch muscles. For instance, GP activity ranges from 20 units in the slow-twitch SOL to 130 units in the fast-twitch white gastrocnemius muscle. Among the fast-twitch red gastrocnemius and plantaris muscles,
GP activity values ranged from 62 to 140, respectively (Noble & Ianuzzo, 1985). This highlights how even among fast-twitch muscles, there is a great deal of variability in GP activity.

<table>
<thead>
<tr>
<th>PFK</th>
<th>LDH</th>
<th>CS</th>
<th>SDH</th>
<th>βHAD</th>
<th>GP</th>
<th>CK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zonderland et al. (1999), (rat)  (units=U/g wt)</td>
<td>EDL=13  SOL=7</td>
<td>-----</td>
<td>EDL=32  SOL=23</td>
<td>-----</td>
<td>EDL=10  SOL=12</td>
<td>-----</td>
</tr>
<tr>
<td>Tesch et al. (1989)  (human)  (units=µmol/g dw min)</td>
<td>-----</td>
<td>VL:  FT=264  ST=154</td>
<td>VL:  FT=15  ST=20</td>
<td>-----</td>
<td>VL:  FT=11  ST=19</td>
<td>-----</td>
</tr>
<tr>
<td>Nemeth et al. (1989)  (rat)  (units =mol/kg dw/hr)</td>
<td>-----</td>
<td>EDL=80-160  SOL=10-30</td>
<td>-----</td>
<td>-----</td>
<td>EDL=1-8  SOL=3-5-9</td>
<td>-----</td>
</tr>
<tr>
<td>Melichna et al. (1987)  (rat)  (LDH units = µkat/g ww; CS/HADH units = nkat/g ww)</td>
<td>-----</td>
<td>EDL=5.3  SOL=3.1</td>
<td>EDL=49  SOL=109</td>
<td>-----</td>
<td>EDL=47  SOL=51</td>
<td>-----</td>
</tr>
<tr>
<td>Noble &amp; Ianuzzo (1985)  (rat)  (units=µmol / g/min)</td>
<td>SOL=20  RG=68  PL=95  WG=118</td>
<td>-----</td>
<td>SOL=30  RG=41  PL=30  WG=13</td>
<td>SOL=3  RG=5  PL=3  WG=2</td>
<td>SOL=10  RG=10  PL=7  WG=3</td>
<td>SOL=23  RG=62  PL=140  WG=130</td>
</tr>
<tr>
<td>Soar et al. (1983)  (rat)  (units=µmol/g ww)</td>
<td>RG=44  VLS=48</td>
<td>-----</td>
<td>RG=12  VLS=3</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Chi et al. (1983)  (human)  (units=mol/kg protein/hr)</td>
<td>-----</td>
<td>VL: Type1=11 Type2=45 Gastrocnemius; Type1=16 Type2=57</td>
<td>VL: Type1=4.8 Type2=2.7 Gastrocnemius; Type1=5.9 Type2=2.7</td>
<td>-----</td>
<td>VL: Type1=6.7 Type2=3.0 Gastrocnemius; Type1=5.6 Type2=2.7</td>
<td>-----</td>
</tr>
<tr>
<td>Gillespie et al. (1982)  (rat)  (units=IU/g ww)</td>
<td>SOL=5  VLP=25  VLS=32</td>
<td>SOL=180  VLP=420  VLS=480</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
</tbody>
</table>

Table 1.2. Differences in oxidative and glycolytic potential among different types of skeletal muscles in both rats and humans. Abbreviations: FT=fast-twitch, SOL=solus, ST=slow-twitch, RG=red gastrocnemius, PL=plantaris, WG=white gastrocnemius, VL=vastus lateralis, VLP=vastus lateralis profundus, VLS=vastus lateralis superficialis.
The same property is also observed in CS activity which ranges from 13 in the white gastrocnemius to 41 in the red gastrocnemius muscle (Noble & Ianuzzo, 1985). This information reinforces the reason why muscle fiber type must be taken into consideration when assessing the effect of hormones since each skeletal muscle has its own level of enzymatic activity which is related to its function.

The availability of substrates for the different metabolic pathways can also differ as follows:

(1) In humans, at rest, glycogen content can be greater in fast-twitch muscles compared to slow-twitch muscles (Bottinelli & Reggiani, 2000; Esbjornsson-Liljedahl et al., 1999; Hochachka, 1994). In rats, information varies as some studies have shown glycogen to be greater in fast-twitch muscles relative to slow-twitch (Azevedo et al., 1998; Carvalho et al., 1997; Welsh & Lindinger, 1993), whereas others have shown no muscle fiber differences (Welsh & Lindinger, 1997; Carvalho et al., 1996). Since glycogen provides the substrate needed for ischemia, muscles with less glycogen will provide less substrate for glycolysis. Glycogen levels were measured in the muscles investigated in this thesis to clarify muscle fiber effects especially since estradiol and progesterone regulate glycogen metabolism (reviewed in Section 1.2.e-5).

(2) At rest, creatine phosphate (CP) is greater in fast-twitch versus slow-twitch muscles. For example, human skeletal muscle studies have reported values of 72 and 83 µmol/g dry weight (dw) in slow-twitch and fast-twitch fibers respectively (Bottinelli & Reggiani, 2000). Similar results were also observed in rats (Carvalho et al., 1997; Welsh & Lindinger, 1997; Carvalho et al., 1996; Welsh & Lindinger, 1993). Fast-twitch muscles, which have higher amounts of CP, also have greater CK activity, whereas slow-twitch muscles, with relatively lower CP levels,
have lower CK activity (Yamashita & Yoshioka, 1991). This suggests that the amount of energy available from CP will be lower in slow-twitch muscles compared to fast-twitch muscles.

(3) In human skeletal muscle, *in vivo* ATP content is relatively similar (22-23 µmol/g dw) between fast- and slow-twitch fibers (Esbjornsson-Liljedahl *et al.*, 1999). However, in rats, ATP levels in fast-twitch muscles are greater than slow-twitch muscles, with fast-twitch muscles ranging between 26-30 µmol/g dw and slow-twitch muscles ranging from 19-22 µmol/g dw (Carvalho *et al.*, 1997; Welsh & Lindinger, 1997; Carvalho *et al.*, 1996). This suggests that species differences in ATP levels based on fiber type need to be considered in the muscle model being used or when comparing different species (i.e., rats versus humans).

Altogether these studies indicate that the fiber types in skeletal muscle are important to consider when investigating the effect of hormones on metabolism, since each fiber type has its own metabolic adaptation. As such, experiments in this thesis were specifically designed to identify differences in muscles with varying fiber types on the metabolic factors studied.

**1.1.h Skeletal Muscle Ischemia – Changing the Flow of Things**

Skeletal muscles have adapted to cope with conditions when there is a variability in blood flow. Ischemia is defined as the reduction in blood flow resulting in reduced oxygen availability to the organ. When this occurs, this reduced availability of oxygen shifts metabolism towards anaerobic pathways that can still generate some ATP, albeit for a limited amount of time due to negative feedback from metabolic pathways. As will be discussed in section 1.2, the proposed studies will investigate the impact that estradiol and progesterone exert on skeletal muscle metabolic response during ischemia. Estradiol and progesterone can alter glucose and glycogen metabolism (reviewed in 1.2.e and 1.2.f). During ischemia, metabolism is limited to anaerobic
sources of energy (ATP) production. Glycolysis is the primary metabolic pathway that converts pyruvate to lactate, thereby generating ATP (see Figure 1.2). Since estradiol and progesterone have the ability to modulate glucose and glycogen metabolism (as reviewed in sections 1.2.d-f), ischemia was studied to understand the implications of these hormonal adaptations. Thus, the following section will briefly summarize the metabolic processes that occur during skeletal muscle ischemia to establish the ischemic experimental model that was utilized.

During global ischemia, there is a lack of perfusion to skeletal muscles which renders them into a state of anaerobiosis. Under these conditions, skeletal muscle can only rely on two key systems for energy production: CP degradation and anaerobic glycolysis (Figure 1.2). As reviewed in Section 1.1.g, skeletal muscles have high CP levels that vary between fast- and slow-twitch muscles. The breakdown of CP is catalyzed by the enzyme creatine kinase (CK) releasing a high energy phosphate from CP to ADP, creating ATP (Figure 1.2). CP is completely utilized after an hour of global ischemia in rats (Carvalho et al., 1996; Blum et al., 1988). Additionally, during anaerobic glycolysis, the conversion of pyruvate to lactate liberates NAD^+, which allows the reaction between glyceraldehyde-3-phosphate (Gly-3-P) to 1,3-bisphosphoglycerate (1,3-BPG) to proceed, thus facilitating ATP production upstream from 1,3-BPG to pyruvate (Figure 1.2). Glycogen is important to anaerobic glycolysis because it is a source of glycosyl units. The breakdown of glycogen is catalyzed by the enzyme glycogen phosphorylase (GP) to release glucose-1-phosphate (G-1P), which is then converted to glucose-6-phosphate (G-6-P) via the enzyme phosphoglucomutase (Figure 1.2).
Figure 1.2. Metabolic pathways active during global ischemia in skeletal muscle. Simplified schematic illustrates the production of ATP via glycolysis, for which glycogen provides the substrate G-6-P and the metabolic end product of this process is lactate. Schematic also illustrates the breakdown of CP to produce ATP during ischemia. ADP = adenosine diphosphate; ATP = adenosine triphosphate; 1,3-BPG = 1,3-bisphosphoglycerate; CK = creatine kinase; DHAP = dihydroxyacetone phosphate; F-1,6-BP = fructose-1,6-bisphosphate; G-6-P = glucose-6-phosphate; Gly-3-P = glyceraldehyde-3-phosphate; GP = glycogen phosphorylase; LDH = lactate dehydrogenase; NAD⁺ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; P_i = inorganic phosphate; PFK = phosphofructokinase.
By 2 hours of ischemia, CP levels are significantly depleted in skeletal muscle (Carvalho et al., 1996). However, skeletal muscle lactate levels continued to increase and plateaued at 3 hours of ischemia (Carvalho et al., 1996). Anaerobic glycolysis is limited by substrate availability and feedback inhibition on PFK activity from increasing cellular acidity. This was illustrated in rat hind limb muscles (*soleus, plantaris, and white gastrocnemius*) that were exposed to hypoxic perfusion without glucose, which allowed for washout of metabolic end products (Welsh et al., 1997). There were greater decreases in glycogen content in muscles that had hypoxic perfusion compared to muscles that were only ischemic and did not have hypoxic perfusion for the same time period (Welsh et al., 1997). Thus, removing metabolic by-products such as lactate and hydrogen ions facilitated glycolysis to continue further because PFK activity was not inhibited.

The 3 hour ischemic period was selected because longer periods of ischemia would lead to cell death. Experiments that investigated tissue necrosis after 3 to 5 hours of ischemia in canine skeletal muscle demonstrated that there was 2% tissue necrosis in muscles exposed to 3 hours of ischemia versus approximately 90% tissue necrosis in muscles exposed to 5 hours of ischemia (Labbe et al., 1987). In rats, 3 hours of ischemia was shown to not significantly alter muscle viability index (MVI) assessed as mRNA degradation in skeletal muscle, but 6 hours of ischemia did not enable the return of MVI back to normal (Akahane et al., 2001). Thus, the 3 hour ischemic period in this thesis was selected to maximize the response of metabolic pathways in skeletal muscle without compromising tissue viability.
1.2 OVERVIEW OF ESTRADIOL & PROGESTERONE ON SKELETAL MUSCLE

1.2.a. What is Known in Postmenopausal Women

The value of hormonal status to skeletal muscle is evident in women as they approach menopause. Menopause is characterized by significant reductions in female sex hormone levels that ultimately result in the cessation of menstruation and the end of female reproductive capacity. Importantly, skeletal muscle structure and function is also altered after menopause (Messier et al., 2011; Maltais et al., 2009). Specifically, a reduction in muscle mass, known as sarcopenia, contributes to the reduction in muscle strength and impairment of day-to-day tasks required for various movements in post-menopausal women (Messier et al., 2011; Maltais et al., 2009). Sarcopenia is the imbalance between muscle protein synthesis, muscle protein breakdown, and increased catabolic factors such as oxidative stress and inflammation (Maltais et al., 2009). Postmenopausal women may develop sarcopenia due to reduced physical activity, reduced protein intake, and increased oxidative stress (Maltais et al., 2009). A systemic review by Greising et al. (2009) examined 23 studies of postmenopausal women published between the years 1987 and 2007 and the impact of hormone therapy on skeletal muscle strength. Overall, they found that 6 of 23 publications showed no effect of hormone therapy on skeletal muscle strength (muscle groups examined included hip abductors, knee flexors, forearm flexors, knee extensors, thumb adductors) whereas the remaining 17 of the 23 studies showed positive effects of hormone therapy on skeletal muscle strength. In addition, there was a small (5%) increase in skeletal muscle strength observed in postmenopausal women who were given hormone therapy compared to those who were not. The contradictory results reported by these studies on whether or not estradiol or combination hormone (both estradiol and progesterone) therapy alters skeletal muscle mass and function in postmenopausal women may be due to the differences in the dosage of hormone treatment, the duration of treatment, the levels of physical activity, diet, or the use of
other medications. The following section will further discuss this topic by highlighting key information from studies examining sex differences in skeletal muscle metabolism, which has been extensively reviewed in the literature (Tarnopolsky, 2008; Tarnopolsky & Saris 2001; Ruby & Robergs 1994).

### 1.2.b. Sex-Related Differences in Baseline Skeletal Muscle Metabolism

To highlight the key findings of studies investigating sex differences in skeletal muscle metabolism, the following will be summarized: (1) what is known during resting conditions in normal sedentary (non-exercised trained) individuals with respect to enzyme activities and baseline metabolism; (2) what is known of sex differences in metabolism from the exercise literature, including what effect the ovarian cycle in women exerts on skeletal muscle metabolism during exercise; (3) what is known about female sex hormones and their effects on the skeletal muscle.

Sex differences in the activity of various metabolic enzymes have been shown to exist between sedentary men and women. A summary of these findings is provided in Table 1.3. In general, most studies took biopsies from the *vastus lateralis* muscle of men and women, with the exception of the study by Apple & Rogers (1986), which took skeletal muscle biopsies from the *gastrocnemius* muscle. This information is important because there are significant metabolic differences among skeletal muscle fiber types and there is evidence that estrogen may affect certain fiber types more than others. In the fast-twitch *vastus lateralis* muscle, the activity of enzymes of glycolysis such as hexokinase (HK) (Simoneau & Bouchard, 1989; Simoneau *et al*., 1985; Green *et al*., 1984), PFK (Simoneau & Bouchard, 1989; Simoneau *et al*., 1985; Green *et al*., 1984), LDH (Simoneau & Bouchard, 1989; Simoneau *et al*., 1985; Green *et al*., 1984), and the enzyme for glycogenolysis GP (Green *et al*., 1984; Komi & Karlsson, 1978) are lower in
women compared to men.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gender Effect</th>
<th>% difference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>M = F</td>
<td></td>
<td>Simoneau &amp; Bouchard (1989)</td>
</tr>
<tr>
<td>HK</td>
<td>M &gt; F</td>
<td>12%</td>
<td>Simoneau &amp; Bouchard (1989)</td>
</tr>
<tr>
<td></td>
<td>M &gt; F</td>
<td>17%</td>
<td>Simoneau et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>M &gt; F</td>
<td>15%</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td></td>
<td>Komi &amp; Karlsson, (1978)</td>
</tr>
<tr>
<td>PFK</td>
<td>M &gt; F</td>
<td>20%</td>
<td>Simoneau &amp; Bouchard (1989)</td>
</tr>
<tr>
<td></td>
<td>M &gt; F</td>
<td>16%</td>
<td>Simoneau et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td></td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Komi &amp; Karlsson, (1978)</td>
</tr>
<tr>
<td>GP</td>
<td>M &gt; F</td>
<td>24%</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td></td>
<td>Komi &amp; Karlsson (1978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simoneau et al. (1985)</td>
</tr>
<tr>
<td>LDH</td>
<td>M &gt; F</td>
<td>35%</td>
<td>Simoneau &amp; Bouchard (1989)</td>
</tr>
<tr>
<td></td>
<td>M &gt; F</td>
<td>32%</td>
<td>Simoneau et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>M &lt; F</td>
<td></td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Komi &amp; Karlsson, (1978)</td>
</tr>
<tr>
<td>SDH</td>
<td>M &lt; F</td>
<td>19%</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td>HAD</td>
<td>M = F</td>
<td></td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td></td>
<td>Roepstorff et al. (2005)</td>
</tr>
<tr>
<td>PK</td>
<td>M &gt; F</td>
<td>18%</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td>CS</td>
<td>M = F</td>
<td></td>
<td>Tarnopolsky et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td></td>
<td>Roepstorff et al. (2005)</td>
</tr>
</tbody>
</table>

*Table 1.3.* Summary of sex differences in the activity of different metabolic enzymes from the fast-twitch *vastus lateralis* muscles of men and women.
Therefore, it seems that glycolytic potential in fast-twitch muscles are lower in women compared to men. However, others have reported no gender differences in PFK and HK activity (Blomstrand et al., 1986; Komi & Karlsson, 1978), or GP activity (Simoneau et al., 1985), and one study reported that LDH activity in women was greater than men (Komi & Karlsson, 1978) despite studying these enzymes in the same muscle (vastus lateralis). These contradictory results may be due to the muscle examined because these studies used a "mixed" muscle fiber type (variable proportions of fast- and slow-twitch fibers), and significant differences in metabolic potential exist in different muscle fibers as discussed in Section 1.1.g.

1.2.c Sex Differences on Substrate Utilization During Exercise

Under metabolic stress, such as exercise, studies have found that sex differences in metabolism exist. During submaximal exercise, women tend to rely more on lipid and less on carbohydrate metabolism compared to men (Isacco et al., 2012; Tarnopolsky & Ruby, 2001; Ashley et al., 2000; Tarnopolsky, 2000; Tate & Holtz, 1998; Horton et al., 1996; Ruby & Robergs, 1994). This information is supported by experiments using the respiratory exchange ratio (RER) which utilizes indirect calorimetry to measure oxygen consumption (VO₂; L/min) and the relative amounts of CO₂ and O₂ in expired gas samples (Ruby & Robergs 1994). The value of RER is that it indicates the amount of carbohydrate use relative to the amount of fat use such that a low RER value indicates a greater reliance on fat metabolism versus carbohydrate metabolism. Tarnopolsky (2008) reviewed sex differences on RER from 25 published studies and found that women have statistically lower RER compared to men (0.87 vs 0.90, P<0.02, respectively). Specifically, after approximately 90 minutes of running exercise, women had lower RER and utilized 25% less glycogen in fast-twitch vastus lateralis muscles relative to men (Tarnopolsky et al., 1990). In individuals who performed 3 bouts of 30 seconds of cycle
sprints, Type I skeletal muscle fibers ATP breakdown products and glycogen consumption were lower in women compared to men (Esbjornssoon-Liljedahl et al., 2002). However, in Type II skeletal muscle these women had smaller reductions in ATP, and less accumulation of ATP breakdown products, compared to men. Thus, this literature strongly supports that sex differences in skeletal muscle must also take into account muscle fiber types. Animal models to test whether estradiol or progesterone modulate these sex effects are discussed in section 1.2.d-f.

1.2.d Ovarian Cycle & Exercise Metabolism

The ovarian cycle in women affects exercise metabolism and has been reviewed in detail by Oosthuyse & Bosch (2010). Studies have typically examined women in the mid-follicular (associated with moderate estradiol and low progesterone levels) and mid-luteal phase (associated with high estradiol, progesterone levels). Differences in skeletal muscle carbohydrate metabolism were noted between these phases of the ovarian cycle in women. Experiments were performed in young women who exercised for 60 minutes in either the follicular and luteal phases (Hackney, 1999). This study demonstrated that in the fast-twitch vastus lateralis muscle, women in the follicular phase used approximately 25% more muscle glycogen than those women in the luteal phase (Hackney, 1999), which supports the previous observations that there is reduced glycolytic metabolism in the luteal phase. Young women aged 20-24 years of age who were in the luteal phase of their menstrual cycle used 25% less glycogen after 90 minutes of submaximal cycling exercise (defined as 65% peak O2 consumption) compared to women in the follicular phase (Devries et al., 2006). As such, differences in muscle glycogen consumption at different phases of the ovarian cycle illustrate the importance of these circulating sex hormones on muscle metabolism.
1.2.e Estradiol & Carbohydrate Metabolism During Exercise

Animal models have demonstrated that estradiol may enhance skeletal muscle glucose metabolism. Generally, estradiol stimulates insulin sensitivity and enhances glycogen storage (Oosthuyse & Bosch, 2010). Glucose tolerance, measured as plasma blood glucose levels before and 30 and 60 min after injection of glucose, was lower in estrogen treated (ET) mice compared to controls, which may indicate increased glucose uptake and an improvement in glucose handling with ET (Carrington & Bailey, 1985). Reduced plasma insulin response to glucose was observed in OVX animals, which could be reversed with ET (Bailey & Ahmed-Sorour, 1980). It is possible that estradiol increased insulin sensitivity since basal skeletal muscle glucose uptake in conjunction with insulin was increased more with estradiol treatment (Puah & Bailey, 1985; Shamoon & Felig, 1974) compared to OVX animals (Kumagai et al., 1993; Puah & Bailey, 1985). Insulin stimulation also increased glycogenesis in all hormone groups (due to greater glucose uptake and conversion of glucose to glycogen), but to a greater degree in the ET group. Insulin sensitivity and glucose tolerance were also shown to differ during the rat ovarian cycle (Bailey & Matty, 1972). Females in proestrus (phase of ovarian cycle when plasma plasma estradiol reaches peak levels) tended to have a greater glucose tolerance and higher plasma insulin levels compared to females in metestrus (phase of ovarian cycle when estradiol is at its lowest). Taken together, these studies may provide evidence that ovarian sex hormones, in particular estradiol, are involved in skeletal muscle glucose metabolism. This is important because glucose can either be shunted into glycolysis or stored as glycogen in skeletal muscle.

Glycogen metabolism in skeletal muscle is also affected by female sex hormones. OVX female animals had reduced skeletal muscle glycogen content, and ET returned skeletal muscle glycogen content beyond intact control values (Carrington & Bailey, 1985; Ahmed-Sorour & Bailey, 1981). During sub-maximal exercise in a rat model, skeletal muscle glycogen content
was significantly reduced in OVX females; however, estrogen treatment attenuated the reduction in muscle glycogen, which was termed the “glycogen-sparing effect” (Kendrick et al., 1987). The same results were reproduced in male rats receiving estradiol under similar experimental conditions (Rooney et al., 1993; Kendrick et al., 1991). As such, estradiol's glycogen-sparing effect in skeletal muscle of females may be an important factor in other metabolic stresses such as ischemia, because during ischemia, glycogen is the greatest substrate for energy production. Changes in how glycogen is utilized is important in the ability of skeletal muscle to maintain its energy levels and the impact of estradiol and progesterone on this will be investigated in this thesis.

1.2.f Progesterone & Carbohydrate Metabolism During Exercise

Some animal studies have reported that progesterone administration alone has a minimal effect on skeletal muscle carbohydrate metabolism. In contrast, when administered in combination with estradiol, progesterone either “antagonized” (by removing the estradiol effect) or did not alter the effects elicited by estradiol. For example, although both estradiol and progesterone treatments in OVX mice increased skeletal muscle glycogen content, the greatest increments were produced with estradiol alone, while the effects of progesterone alone were comparatively small (Carrington & Bailey, 1985; Ahmed-Sorour & Bailey, 1981). Combination of both estradiol and progesterone resulted in smaller increases of tissue glycogen content than estradiol alone, indicating an antagonistic effect of progesterone on glycogenic effects of estradiol. How the mechanism by which progesterone antagonized the effect of estradiol in this process is not clear and have yet to be studied.

In both the presence and absence of insulin, progesterone has been reported to impair glucose metabolism in skeletal muscles of female rats (Sutter-Dub, 1986). Specifically, in OVX
rats, insulin sensitivity was reduced compared to intact controls and progesterone administration alone did not change this; however, estradiol treatment or combined estradiol and progesterone treatment restored insulin sensitivity to intact animal values (Sutter-Dub, 1986). In OVX rats, glycogen synthesis and glucose uptake were reduced compared to intact females (Kumagai et al., 1993). Progesterone administration did not modify this effect, whereas estradiol increased these variables. Additionally, combined administration of estradiol and progesterone restored glycogen synthesis and glucose uptake to similar levels as observed in gonadally intact rats (Kumagai et al., 1993). In another study where progesterone was administered with estradiol, progesterone antagonized the effects of estradiol on insulin-stimulated glucose uptake and glycogenesis in the slow-twitch SOL muscle (Puah & Bailey, 1985). One *in vitro* study showed that progesterone inhibited glycogen synthesis and the rate of glycolysis in the fast-twitch EDL, whereas in the slow-twitch SOL, progesterone only inhibited the rate of glycolysis (Leturque et al., 1989). Thus, progesterone's primary effect on carbohydrate metabolism appears to oppose that of estradiol in skeletal muscle. The role of skeletal muscle fibers is important since fast-twitch and slow-twitch differences in response to progesterone seem to exist. Under ischemic conditions, progesterone may predispose skeletal muscle to reduced anaerobic glycolysis due to reduced glycogen levels, providing less substrate for glycolysis, generating less energy (ATP) as a result.

**1.2.g. Female Sex Hormones on Skeletal Muscle Enzyme Activity**

Earlier in Section 1.2.b, sex differences in enzyme activity were identified, and these differences can be attributed to estradiol. In one study, OVX reduced CK activity in various skeletal muscles (*vastus lateralis, gracilis, gastrocnemius, soleus*) (Ramamani et al., 1999), and estradiol treatment for 30 days restored CK activity to control values. In another study, the effect of estradiol treatment was examined in *vastus lateralis* muscles of female rats given estradiol for
21 days (Beckett et al., 2002). The latter study showed that glycogen synthase fractional velocity (defined as the ratio of glycogen synthase activity at physiologic G-6-P concentration to total activity at saturated G-6-P concentrations) of OVX females was reduced, but returned to intact values with estradiol treatment (Beckett et al., 2002). They also reported that PFK and β-hydroxyacyl-CoA dehydrogenase (βHAD) activity were not altered by hormone manipulation. However, CS activity was increased by estradiol treatment compared to OVX females.

It is important to note that muscle fiber differences in βHAD activity have been reported, which can be attributed to the types of skeletal muscles studied. In the fast-twitch white gastrocnemius muscle, βHAD activity was not affected by estradiol treatment (Campbell et al., 2001). The white gastrocnemius muscle has a high proportion of Type IIb fibers (Appendix 1) indicating a higher dependence on glycolytic sources of energy production and reduced oxidative capacity. It is theorized that estradiol did not have an effect on βHAD activity since it has a lower oxidative potential (as indicated by CS activity levels summarized in Appendix 1). In contrast, in the red gastrocnemius muscle, OVX reduced βHAD activity by 20%, and estradiol treatment returned βHAD activity to control values (Campbell et al., 2001). Progesterone treatment did not alter βHAD activity compared to OVX and blocked the effect of estradiol (Campbell et al. 2001). The red gastrocnemius has approximately 50% Type I and 50% Type II fibers, of which approximately 35% are Type IIa fibers (Appendix 1). Thus, in the fast-twitch 'red' gastrocnemius muscle, female sex hormone effects were more clearly illustrated, which can be attributed to their greater reliance on oxidative sources of energy (indicated by CS activity levels summarized in Appendix 1). When looking at citrate synthase (CS) activity, an indicator of the Citric Acid Cycle, estradiol treatment increased CS activity in the fast-twitch vastus medialis, but did not affect CS activity in either the white or red gastrocnemius (Campbell & Febbraio, 2001). Thus, deciphering the role of female sex hormones on the activity of various
metabolic enzymes in fast-twitch skeletal muscle requires clarification. Even among fast-twitch muscles, female sex hormones effects are varied and are most likely dependent on the metabolic adaptation of that muscle. This premise was examined in Study 1 which investigated the role of estradiol on key regulatory enzymes of metabolism.

1.3 THE OVARIAN HORMONES ESTRADIOL & PROGESTERONE

It is evident that estrogen and progesterone have a significant impact on skeletal muscle metabolism. Understanding the mechanism by which these sex hormones mediate their cellular effects will enhance our knowledge of how estradiol and progesterone affect skeletal muscle. The first part of this section will review the synthesis of estradiol and progesterone, followed by the mechanism of how estradiol impacts target cells and induces cellular changes via estrogen receptors (ER).

1.3.a. Ovarian Hormones: Biosynthesis

In females, the ovary is the main organ that produces the hormones estradiol and progesterone. These steroid hormones are produced in specialized ovarian structures known as the follicle and corpus luteum (Griffin & Ojeda, 2000; Guyton & Hall, 1997). The follicle is composed of granulosa cells and thecal cells that surround the ovum. Post ovulation, the residual cells of the ovulated follicle undergo structural transformations to form the corpus luteum. Estradiol and progesterone are synthesized from a common initial precursor, cholesterol (Figure 1.4) (Griffin & Ojeda, 2000; Knobil & Neill, 1998). Cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme complex. Pregnenolone is converted to progesterone via the enzyme 3β-hydroxysteroid dehydrogenase. As illustrated in Figure 1.4, a series of chemical reactions lead to the formation of estrone which can be converted
into estradiol via the enzyme 17β-hydroxysteroid dehydrogenase. Testosterone can also be converted to estradiol via the aromatase enzyme. The granulosa cells synthesize estradiol from androgens produced by the thecal cells. Progesterone is mainly produced by the corpus luteum post ovulation; however, the granulosa cells of the preovulatory follicle synthesize and secrete progesterone which coincides with the LH surge.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) produced by the pituitary control estradiol and progesterone synthesis (Griffin & Ojeda, 2000; Guyton & Hall, 1996; Knobil & Neill, 1998). As illustrated in Figure 1.4, LH and FSH can stimulate progesterone secretion; however, only LH promotes androgen secretion. Estradiol synthesis is dependent on LH and FSH. LH stimulates the production of the androgen androstenedione in the thecal cells which can diffuse to the granulosa cell to be converted to estradiol. FSH stimulates the production of estradiol in the granulosa cells by directly activating the aromatase enzyme complex that catalyzes the conversion of testosterone to estradiol. The “two cell-two gonadotropin” hypothesis illustrated in Figure 1.4 identifies that the follicular theca is regulated by LH and synthesizes androgens which diffuse to the granulosa cell compartment to be converted to estradiol and the aromatase enzyme that catalyzes this reaction is under FSH regulation (Griffin & Ojeda, 2000; Knobil & Neill, 1998; Armstrong, & Dorrington, 1977).
Figure 1.3. The synthesis of estradiol and progesterone from cholesterol. 1 = Cholesterol side-chain cleavage (SCC) enzyme complex, 2 = 3beta-hydroxysteroid dehydrogenase (3B-HSD), 3 = 17alpha-hydroxylase, 4 = 17,20-lyase, 5 = aromatase, 6 = 17beta-hydroxysteroid dehydrogenase. Reproduced with permission from Griffin & Ojeda, Textbook of Endocrine Physiology, pg 188, 2004.
Figure 1.4. LH and FSH regulation of estradiol and progesterone synthesis in the thecal and granulosa cells of the ovary. SCC = Cholesterol side-chain cleavage enzyme complex, 3B-HSD = 3beta-hydroxysteroid dehydrogenase. Reproduced with permission from Griffin & Ojeda, Textbook of Endocrine Physiology, pg 190, 2004.
1.3.b. Overview of Steroid Hormone Receptors

Estradiol and progesterone's effects are mediated by binding to a hormone receptor in the target cell. These receptors belong to the steroid hormone/nuclear receptor superfamily of enhancer proteins. The steroid hormone receptors contain specialized domains that serve specific functions in the steps from the hormone binding to the receptor to gene transcription. Estrogens and other steroid hormones are hydrophobic molecules that easily diffuse across cell membranes. In general, the steroid hormone receptors undergo a conformational change upon binding to the hormone (ligand) which causes the receptor to become dissociated from heat shock and other chaperone proteins (hsp). The steroid hormone receptor bound to its ligand is also phosphorylated, which results in receptor activation. This 'activated' hormone/receptor complex can dimerize with other hormone/receptor complexes and together, localize to the cell nucleus, and bind to hormone response elements located on the DNA to initiate gene transcription. The initiation of gene transcription is a complicated, multi-step process requiring many proteins. Once the hormone/receptor dimer is bound to the hormone response element, it will interact with co-activators that stabilize transcription factor binding and promote the assembly of the transcription initiation complex.

1.3.c. ER Structure and function

In skeletal muscle, ER have been identified (as reviewed in 1.3.h), but reports on progesterone receptor expression (PR) in skeletal muscle are varied. In women, the levator ani muscle reported PR expression (Copas et al., 2001), but PR mRNA was undetectable in neck and shoulder muscles of heifers (Pfaffl et al., 2002). As such, the focus of this section will pertain to ER because their role in modulating skeletal muscle metabolism was investigated in this thesis. In general, there are 5 domains in the ER:
**A/B domain** (amino acids 1-180) which is also called the activation function 1 (AF-1) domain, carries out protein-protein interactions and transactivation, and does not require ligand binding to function (Ellmann et al., 2009).

**C-domain** (amino acids 181-263) contains the DNA-binding domain which lies towards the center of the ER and has 9 conserved cysteine residues that provide the ER with sequence-specific DNA activity; 8 of these cysteine residues are conserved because of their role in binding to zinc (Zn) and are also known as the 'Zn-finger domains'. It is this part of the ER that binds to specific regions on DNA sites which are known as estrogen response elements (ERE) which has the consensus sequence 5'-AGGTCA
NNN
TGACCT-3' and enables the ER to bind to this site as a dimer. The estradiol/ER complex can form dimers through this domain either as a homodimer (ERα/ERα, ERβ/ERβ) or as other heterodimer (ERα/ERβ).

**D-domain** (amino acids 264-302) is also known as the hinge-region that separates the DNA-binding domain from the ligand binding domain, and has sequences that facilitate receptor dimerization and nuclear localization. The hinge region can also interact with co-repressor proteins (Klinge, 2000).

**E-domain** (amino acids 303-552) is also known as the activation function 2 (AF-2) domain, facilitates ligand binding, and is an interaction site for co-activators and co-repressors. It is the second most highly conserved region of the ER and lies near the carboxyl terminus and folds into a structure that consists mostly of α-helices that form a hydrophobic ligand-binding pocket. The ligand-binding domain also participates in ER dimerization. The structure of the ligand binding
domain is composed of 11 to 12 α-helices and 1 β-sheet (Mueller-Fahrnow & Egner, 1999). Various conformational changes occur in the ligand binding domain, which depending on type of ligand present, alters the position of α-helices. In addition, AF-2 is positioned to allow co-activators to bind and initiate transcription.

**F-domain** (amino acids 553-595) represents the last 45 amino acids in ERα and approximately the last 30 amino acids in ERβ. It is thought that the purpose of this domain is to restrain dimerization of ER, particularly in the beta subtype. The F domain may distinguish ER agonists from antagonists (Klinge, 2000).

### 1.3.d. ER Isoforms

There are 2 isoforms of the ERs, alpha (ERα) and beta (ERβ), which bind to estradiol (Figure 1.6). These isoforms have different functions and illicit different effects of estradiol in a cell-specific manner. The relevance of ER isoform effects in skeletal muscle is reviewed in 1.3.h.

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**Figure 1.5.** Schematic structure of the two ER isoforms and the percent homology between them. Information was collected from Klinge (2000) and Ellmann *et al* (2009). The 5 domains of the ER are indicated in different colours. The percent homology between ERα and ERβ was taken from Pace *et al* (1996).
1.3.e. Co-activators of the ER

When ligand binds to the nuclear receptor in the AF-2 domain, it initiates the activation of gene transcription. There are additional proteins called co-activators that are critical to this activation, and a few of which are discussed below.

The family of p160 proteins are known to bind to the AF-2 region of nuclear receptors in a hormone dependent manner (Edwards, 2000). Also known as steroid receptor co-activators (SRC), three members exist:

(1) SRC-1 (or F-SRC-1 or NCoA-1)

(2) SRC-2, which consists of GRIP-1 (glucocorticoid receptor interacting protein-1) and TIF-2 (transcriptional intermediary factor-2)

(3) SRC-3, cloned independently by several groups and termed either p/CIP, RAC3, ACTR, TRAM-1, or AIB 1. These p160 co-activators do not have DNA-binding activity. They are recruited to the promoters of steroid responsive target genes via protein-protein interaction with nuclear receptors, binding directly to AF-2. These proteins are well-characterized and are important in nuclear receptor mediated gene transcription, and studies from ER antagonists that inactivate AF-2 demonstrate that they do not permit p160 co-activator binding. All SRC members share about 40% sequence homology, and are modular proteins with regions of conserved sequences that have distinct functional domains (Edwards, 2000).

The amino acid residues of ERα helices 3, 4, 5, and 12 are involved in ERα-SRC-1 or ERα-SRC-2 interaction. It is suggested that cell-specific regulation of estrogen on gene expression may be the result of different levels of co-activators/co-repressors in target cells (Klinge, 2000). In addition, differing levels of ERα, ERβ, and ligand may explain how different target cells concisely control gene transcription.
The p160 co-activators have histone acetyltransferase activity (HAT) that result in the acetylation of lysine residues on histone subunits (that have a positive charge). The neutralizing effect decreases the affinity between histone and DNA (that has a negative charge) (Edwards, 2000). This relaxation of the chromatin structure allows access to the transcriptional machinery (RNA polymerase II). Nuclear receptors like ERα are also acetylated by HAT and it is suggested that this functions to repress its transcriptional activity (Wang et al., 2011). Nuclear receptors recruit at least 3 different classes of co-activators that possess intrinsic HAT activity, including p160 (SRC-1 and SRC-3), CBP (p300 cAMP response element binding protein), and CBP associated factor p/CAF (Edwards, 2000). CBP is a secondary co-activator that requires the presence of p160 for co-activation effects on nuclear receptors - CBP together with pCAF affect nuclear receptors by indirect association with p160 (Edwards, 2000) and mediated changes in gene expression.

1.3.f. Co-repressors of the ER

Co-repressors generally function to suppress transcription (Gruber et al., 2002). Generally, co-repressors function by recruiting silencing factors that bind to the DNA, which results in gene silencing (Baniahmad, 2005). In addition, ER antagonists are also capable of promoting the association of the following co-repressors:

(1) NCOR (nuclear receptor co-repressor)

(2) SMRT (silencing mediator for retinoid and thyroid receptor)

Co-repressor binding can be stabilized by hormone antagonists (Baniahmad, 2005). One mechanism for how antagonists achieved receptor blocking is by orientating AF-2 to allow for co-repressor binding and not co-activator binding (Baniahmad, 2005).
1.3.g. Non-Genomic Mechanisms of the ER

There has been much discussion and evidence that rapid non-genomic responses (defined as not directly influencing gene expression as per Losel & Wehling, 2003) to estradiol exist, adding to the complexity of how this sex hormone functions in target cells. The literature indicates that there are plasma membrane-bound ER, known as the G protein-coupled receptor-30 (GPR-30), that are responsible for these non-genomic responses to estradiol in cancer and vascular cells (Barton 2012; Funakoshi et al., 2006; Pedram et al., 2006; Sak & Everaus, 2004). Estradiol can activate several cell-signaling pathways via GPR-30: (1) MAPK family, such as Src and Shc, are induced by ERα to cause Shc/Src/Ras/ERK activation; (2) growth factors, such as IGF1-R and EGFR, can also be activated by estradiol and explain the cross-talk between ER and growth factor receptor signaling; (3) PI3K/AKT signaling pathway may also be activated and explain the role of ER in rapid endothelial nitric oxide release in vascular cells (Acconcia & Kumar, 2006). To date, GPR-30 expression has not been demonstrated in skeletal muscle and what role it may have on its metabolism in different muscle fiber types has yet to be elucidated.

1.3.h. ER Isoform Expression in Skeletal Muscle

Estrogen receptors (ER) are expressed in skeletal muscle (Lemoine et al., 2002; Sauerwein & Meyer, 1989; Meyer & Rapp, 1985; Saartok, 1984; Dahlberg, 1982) which highlights the importance of this sex hormone's non-reproductive role in the body. The ER in skeletal muscle have been reported in rats (Lemoine et al., 2002; Dahlberg, 1982; Dionne et al., 1979; Dube et al., 1976) and humans (Lemoine et al., 2003). As early as 1984, there was evidence that there were more estradiol binding sites in slow-twitch SOL muscle of rabbits compared to fast-twitch gastrocnemius/plantaris muscles (Saartok, 1984).
Two isoforms ERα (Lemoine et al., 2002a&b; Lemoine et al., 2003) and ERβ (Wiik et al., 2003) exist, but ERα mRNA expression is significantly greater (~180 fold) compared to ERβ expression β (Wiik et al., 2003). The link between ER and its functional significance in terms of skeletal muscle metabolism has yet to be fully elucidated. Barros et al. (2009) investigated GLUT4 regulation in the fast-twitch gastrocnemius muscle of ER knockout (KO) α and β female mice and found that GLUT4 expression was reduced significantly in ERKOα mice, but not in ERKOβ mice relative to the wild-type controls. A review by Barros et al. (2011) indicated that ERβ suppresses GLUT4 expression in skeletal muscle. Based on this information, the ERs have a significant role in skeletal muscle glucose metabolism, but muscle fiber type effects are unclear. ER effects on glucose uptake can affect other metabolic pathways such as glycogen metabolism since glucose can either be stored as glycogen or enter glycolysis.

1.3.i. ER Antagonist ICI 182,780

ICI 182,780 (ICI) is a ‘pure’ ER antagonist that blocks both ER isoforms (Howell et al., 2000; Wakeling, 2000; Kuiper et al., 1998; Kuiper et al., 1997; Tremblay et al., 1997). The binding of ICI results in the termination of estradiol effects in target tissues such as breast cancer cells (Howell, 2006). ICI can achieve its effects by the following: (1) block dimerization of ER, (2) block nuclear ER localization, (3) decreases cellular levels of ER content, (4) blocks ER-mediated gene transcription. Thus in this thesis, ICI was used to investigate the effect of inhibiting ER function in skeletal muscle metabolism. Interestingly, there is evidence that ICI can alter ischemic responses in tissue. In rat liver, indices of ischemia/reperfusion injury were greater in the liver of rats treated with ICI 182,780 compared to controls and estradiol treated rats (Eckoff et al., 2002). This suggests that ICI is useful in determining ischemic metabolic responses in skeletal muscle ischemia.
1.3.j. Selective ER Modulator (SERM) Raloxifene

The SERM Raloxifene (RLX) differs from the antagonist ICI as it exhibits both agonist and antagonist effects depending upon the tissue (Dutertre & Smith, 2000). RLX has antagonistic properties in reproductive tissues, but in non-reproductive tissues has agonist effects. RLX has similar effects as estradiol in bone, blood vessels, myocardium (Ogita et al., 2002), and lipid metabolism (Mijatovic et al., 1999). In human skeletal muscle cells, RLX increased mRNA of GLUT4 similar to that of estradiol (Dieli-Conwright et al., 2009), which made it a suitable SERM to test in this thesis.

1.3.k. ER Binding Properties

Estradiol is not the only ligand that binds ER. Although estradiol has the highest affinity for the ER, other estrogens such as estrone and estriol also bind, but with lower affinity (Table 1.5). Pharmaceutically derived ligands such as the SERM Raloxifene and Tamoxifene and the pure ER antagonist ICI 182,780 also bind ER.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>RBA ERα (%)</th>
<th>RBA ERβ (%)</th>
<th>RBA ERα/ERβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>estradiol&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>estrone&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>10</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>estriol&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>11</td>
<td>35</td>
<td>0.3</td>
</tr>
<tr>
<td>Raloxifene&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>50</td>
<td>14</td>
<td>3.6</td>
</tr>
<tr>
<td>Tamoxifene&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>3</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>ICI 182,780&lt;sup&gt;▲&lt;/sup&gt;</td>
<td>45</td>
<td>35</td>
<td>1.3</td>
</tr>
<tr>
<td>Progesterone&lt;sup&gt;■&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.4. Relative binding affinities (RBA) of various ligands to ER α and β. Data from: <sup>▲</sup> Zhu et al. (2006), <sup>■</sup>Kuiper et al. (1997).
1.4 RATIONALE

When looking at sex differences, skeletal muscle metabolic responses vary between males and females, suggesting a potential role for sex hormones in regulating skeletal muscle metabolism (Tarnopolsky, 2008; Devries et al., 2006; Ashley et al., 2000). In women, the loss of ovarian sources of estradiol and progesterone are more apparent as they become menopausal, reaffirming that these hormones are important to many cellular functions in the body. Skeletal muscle strength, which is reduced in post-menopausal women, was improved by hormone therapy. In animal studies, estradiol demonstrates a variety of characteristics that overall favours lipid metabolism and reduces carbohydrate metabolism, which becomes apparent during exercise. A trademark of estradiol is its ability to induce a glycogen-sparing effect during sub-maximal exercise while promoting lipid oxidation. Studies examining progesterone effects in skeletal muscle are not as abundant as estradiol. From what is known, progesterone's effects on carbohydrate and lipid metabolism are opposite to estradiol. For example, glycogen content was reduced in slow-twitch soleus (SOL) muscle strips exposed to progesterone via reductions in glycogen synthesis (Gras et al., 2007) versus that of estradiol which increased glycogen content in the SOL (Carrington & Bailey, 1985; Ahmed-Sorour & Bailey, 1981). The presence of skeletal muscle ER in humans (Lemoine et al., 2003; Wiik et al., 2003), rats (Lemoine et al., 2002a; Lemoine et al., 2002b; Dahlberg, 1982), and bovine (Bechet et al., 1986) reaffirms the significance of estradiol’s effects in skeletal muscle. Although there is not much information addressing the molecular effects of ER pathways in skeletal muscle, evidence suggests that estradiol can act as a mediator of its effects at the nuclear level, altering gene expression of metabolic enzymes. Studies in breast cancer cell lines have shown that creatine kinase (CK) (Crombie et al., 1994; Wang et al., 2002) and lactate dehydrogenase (LDH) (Li et al., 2004) may be under estrogenic control because the promoter regions for their genes contain imperfect
palindromic or half estrogen-response elements (ERE), respectively. The ERα isoform positively mediates glucose uptake in slow-twitch SOL and fast-twitch EDL muscles, reaffirming the role of ER in skeletal muscle glucose metabolism (Gorres et al., 2011).

What role these estrogenic effects will have in skeletal muscle when blood flow is compromised is unknown, especially with respect to ischemia since anaerobic metabolism is the primary source of energy production. It can be speculated that estradiol may inhibit the use of skeletal muscle glycogen reserves (since glycogen-sparing due to estrogen was observed in exercise studies), thus potentially limiting lactic acidosis. On the other hand, the lack of sex hormones (estradiol, progesterone) could increase glycogen use, resulting in increased lactic acidosis. Additionally, there may be a trade-off as to how energy (ATP) levels are sustained during ischemia since reduced anaerobic glycolysis during ischemia would produce less ATP. These issues have yet to be clarified in skeletal muscle. Therefore, what role estradiol will have during ischemia needs to be clarified in order to better understand the balance between reduced anaerobic glycolysis and energy levels. Additionally, what role progesterone has is equally important, particularly since hormone treatment (combination estradiol + progestin) in post-menopausal woman and its effects on ischemic skeletal muscle is virtually unknown.

Since skeletal muscle is a heterogeneous tissue that has varying proportions of slow- and fast-twitch muscle fibers, and fiber specific effects have been noted, studies were conducted in skeletal muscles classified as slow-twitch (Type I) and fast-twitch (Type II). For instance, slow-twitch muscles (or Type I) rely primarily on aerobic metabolism (beta-oxidation, Kreb's cycle, electron transport chain) for energy production. Among fast twitch fibers, there are primarily two sub-types: Type IIa that are adapted to produce energy via aerobic and glycolytic pathways, and Type IIb that rely primarily on glycolytic and anaerobic pathways for energy production. This is especially important because enzymatic activity regulated by estradiol may occur in some
muscles and not others. Evidence for this is illustrated in β-hydroxyacyl-CoA dehydrogenase (βHAD) activity, a key regulator enzyme of beta-oxidation in fat metabolism. Some studies have shown that this enzyme is regulated by estradiol in some fast-twitch muscles (Campbell & Febbraio, 2001), but not in others (Campbell & Febbraio, 2001; Beckett et al., 2002). Given the range of fiber-types that co-exist in fast-twitch muscles, it is possible that the effect of estradiol may be “diluted” by the effect of other fiber types found in that muscle. Therefore, it is important to examine muscles that have specific distributions of skeletal muscle fiber types. The first experimental study in this thesis will focus on clarifying the role of estradiol on key regulatory enzymes of metabolism in different fast-twitch fibers since there are conflicting reports in the literature. The second experimental study will focus on the slow-twitch SOL and fast-twitch EDL muscles to clarify the muscle fiber differences in glucose metabolism due to estradiol and progesterone and test these adaptations with an ischemic model. The third study will also study the SOL and EDL to identify the role of ER on glucose metabolism and test these adaptations with an ischemic model.

The goal of this PhD work is to clarify how estradiol and progesterone effect skeletal muscle metabolism in fast- and slow-twitch muscles under normal and ischemic conditions and identify the mechanisms for these effects. This will clarify the literature's understanding of how these key female sex hormones influence the dynamics of skeletal muscle metabolism and their role under metabolic stress (ischemia).
1.5 HYPOTHESES

1. Estradiol will decrease the activity of enzymes involved in glycogenolysis, glycolysis, and anaerobic glycolysis, not affect aerobic enzymes, and this will occur to a greater extent in skeletal muscles with higher fast-twitch composition.

2. a) Estradiol will increase in vivo glycogen and CP levels to a greater extent in fast-twitch muscles compared to slow-twitch muscles, via the ER.

   b) During ischemia, estradiol will decrease anaerobic glycolysis (lactate accumulation) and energy levels (ATP) because of decreased glycogenolysis due to lowered glycogen phosphorylase activity, which will be mediated by the ER and occur to a greater extent in fast- versus slow-twitch muscles.

3. a) Progesterone will decrease in vivo glycogen levels to a greater extent in fast-twitch muscles compared to slow-twitch muscles.

   b) During ischemia, progesterone will decrease anaerobic glycolysis (lactate accumulation) and energy levels (ATP) because of decreased glycogenolysis due to lower glycogen availability, and in fast- versus slow-twitch muscles.

4. Progesterone will inhibit the metabolic effects of estradiol when given in combination, and this will occur to a greater extent in fast-twitch muscles compared to slow-twitch muscles.
CHAPTER 2

MATERIALS and

METHODS
2.1. Animals

Female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories either as pre-pubertal ovariectomized (OVX) or sham-operated (SHAM) at 3 weeks of age. All rats were housed individually in cages in a room environmentally controlled for temperature (20°C) and light (12 h light-dark cycle), and fed *ad libitum* on commercially available chow (Purina Rat Chow 5001, Ralston Purina Canada Inc., Chomedy, Quebec) and water. A week after procurement, females were allocated into specific groups for each of the main studies (detailed below). All animals were treated in a humane manner and in accordance with the principles of the National Institutes of Health (National Academy of Sciences, 2011) and guidelines of the Canadian Council on Animal Care (Olfert et al., 1993).

2.2. Hormone Manipulation Models

2.2.a. Study 1: Sham-operated (INT, n=5) and OVX (n=12) females were used to study the effects of female sex hormones on enzymatic activity in 3 different fast-twitch muscles (*gastrocnemius, semitendinosus* (SMT), *extensor digitorum longus* (EDL)) (refer to Appendix 1 for further details on fiber type proportions of these fast-twitch muscles). OVX females were randomly allocated to receive 120 days of treatment as follows: (1) placebo (OVX, n=6) or (2) 17β-estradiol (ET, n=6) (Figure 2.1.a.). ET was administered using a 5 mg 17β-estradiol 60 day release pellet (Innovative Research of America, Sarasota, FL) and OVX rats received a placebo pellet. All pellets were inserted subcutaneously behind the neck (as described in next paragraph) every 60 days, for a total of 120 days of treatment (total 2 pellets administered/rat, see Figure 2.1.a). SHAM rats were exposed to the same pellet implantation protocol but did not receive a placebo pellet.
Rats were anesthetized using Isoflurane (5% mg/kg) and once at the proper plane of anesthesia (absence of pain reflexes), the area behind the neck was shaved and disinfected with iodine, and a full thickness 1cm skin incision was made with a sterile scalpel blade. Through the incision using a sterilized trochar, the pellet was tunneled subcutaneously. The skin was stapled closed with a multi-directional release skin stapler (Proximate Plus MD, Ethicon Endo-Surgery, Inc, Cincinnati, OH).

**Figure 2.1.a.** Timeline for the pellet protocol for female rats in Studies 1, 2, and 3. In total, each female received two 60 day pellets (exception: IE+P females received total of 4 pellets, see section 2.2.b) containing the designated hormone/drug for a total treatment time of approximately 120 days. The first pellet was given on Day 1 and 60 days later, each female was given a 2nd pellet (Day 61). On Day 121, all females underwent the final acute experimental protocol described in section 2.3.

Rats were anesthetized using Isoflurane (5% mg/kg) and once at the proper plane of anesthesia (absence of pain reflexes), the area behind the neck was shaved and disinfected with iodine, and a full thickness 1cm skin incision was made with a sterile scalpel blade. Through the incision using a sterilized trochar, the pellet was tunneled subcutaneously. The skin was stapled closed with a multi-directional release skin stapler (Proximate Plus MD, Ethicon Endo-Surgery, Inc, Cincinnati, OH).

**Figure 2.1.** Animal groups used in Study 1.
2.2.b. Study 2: OVX (n=24) females were used to study the effects of estradiol and progesterone on \textit{in vivo} and ischemic skeletal muscle metabolism in two different skeletal muscles, the predominantly fast-twitch EDL and the predominately slow-twitch SOL. OVX females were randomly allocated to receive 120 days of treatment as follows: (1) placebo (OVX, n=6), (2) intermittent 17β-estradiol (discussion for this model of estradiol treatment explained further below) (IE, n=6), (3) progesterone (P, n=6), (4) intermittent 17β-estradiol and progesterone (IE+P, n=6) (Figure 2.2). IE was administered using a 17β-estradiol 14 day release pellet (5 mg, Innovative Research of America, Sarasota, FL) and P was given using a 60 day release progesterone pellet (200 mg, Innovative Research of America, Sarasota, FL). All pellets were inserted subcutaneously behind the neck (as previously described) every 60 days, for a total of 120 days of treatment (total 2 pellets administered/rat, see Figure 2.1.a). The pellets were surgically inserted as described for Study 1 with the exception of the progesterone pellet where, due to the larger size of the progesterone pellet, subcutaneous blunt dissection was required to create a sufficient pocket for pellet insertion. IE+P group were simultaneously administered the 5 mg IE pellet and 200 mg P pellet which were placed in separate locations.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure22}
\caption{Animal groups used in Study 2.}
\end{figure}
**Model for IE:** The estradiol pellets were originally ordered from Innovative Research of America to release a daily dose of estradiol of ~50 pg/ml (high-end of physiologic range) in a 5 mg 17β-estradiol 60 day release pellet (Innovative Research of America, Sarasota, FL). However, after performing the experiments and analyzing plasma estradiol levels, all of the females used in Study 2 had undetectable plasma estradiol levels at the end of 120 days (Appendix 3). To determine what caused these unforeseen results, 4 female Sprague-Dawley rats underwent the usual pilling protocol for 120 days. Blood samples (tail vein) were taken from each female on specified days (Figure 2.3). Plasma estradiol peaked 2 weeks after pilling in all females, and was either very low or undetectable thereafter (Figure 2.3). Although this model of estradiol treatment was not originally designed for the experiments of Study 2, biochemical analyses of these muscles indicated significant biological effects of estradiol. Therefore, this model of estradiol administration was termed "intermittent-estradiol (IE)".

**Figure 2.3.** Plasma estradiol levels in four female Sprague-Dawley rats illustrating dosing regimen of IE pellets. † Second estradiol pellet given 60 days after first estradiol pellet. Rats not represented at each time point had plasma estradiol levels that were not detectable (< 8 pg/ml).
2.2.c. Study 3: OVX (n=18) females were used to study the role of the ER on \textit{in vivo} and ischemic skeletal muscle metabolism in the predominately fast-twitch EDL and predominately slow-twitch SOL muscles using the ER antagonist ICI 182,780 and the SERM (agonist in skeletal muscle) Raloxifene. OVX females were randomly allocated to receive 120 days treatment: (i) placebo (OVX, n=6), (ii) ICI 182,780 (ICI, 25 mg/pellet, 60 day release; Innovative Research of America, Sarasota, FL), or (iii) Raloxifene (RLX, 50 mg/pellet, 60 day release; Innovative Research of America, Sarasota, FL) (Figure 2.4). Pellets were inserted surgically as described for progesterone in Study 2. All pellets were inserted subcutaneously behind the neck (as previously described) every 60 days, for a total of 120 days of treatment (total 2 pellets administered/rat, see Figure 2.1.a).

OVX females were studied as the majority of circulating estradiol was removed in this group. ICI was given to OVX to ensure that any ER effect was blocked completely so that no role of ER was possible. RLX is a chemical agonist to ER and it was shown to mimic estradiol's metabolic effects in skeletal muscle (see section 1.3.j.).

\textbf{Figure 2.4.} Animal groups used in Study 3.
2.3. Final Acute Experimental Model

2.3.a. Surgical Preparation

At the end of the hormone treatment duration of 120 days, all rats were weighed (body weight, bw). Rats were anesthetized with an intraperitoneal injection of Inactin (sodium thiobutabarbitral, 0.1 mg/g bw; Research Biochemicals International, Natick, MA). Maintenance injections (approximately 0.25 to 0.5 ml) of Inactin were given to maintain the plane of anesthesia (Plane 3, described as significantly reduced muscle tone, absent reflex responses, absent pupil response; Dripps et al., 1997). To maintain normal blood gases, the rats were intubated and ventilated with medical air using a rodent ventilator (Kent model RSP1002, Kent Scientific Corp., Litchfield, CT) (refer to Appendices #2 for blood gas data). The right carotid artery was cannulated using PE50 tubing and connected to a pressure transducer to assess hemodynamic data (heart rate, blood pressure) using Biopac Systems model MP100 and AcqKnowledge software (Biopac Systems Inc., Goleta, CA). A dose of 100 IU of heparin (Heparin Leo, Leo Pharma Inc., Ajax, ON) was administered to each rat to prevent any blood clotting. Body temperature was measured with a rectal thermometer and maintained in a range of 37-38°C using a heating pad. Prior to the first \textit{in vivo} biopsy, a 1 cc blood sample was collected in heparinized syringes, spun at 15000g in a pre-cooled 0°C centrifuge, and the plasma was removed and stored at -80°C for plasma estradiol analyses.

As part of the surgical preparation, the skeletal muscles required for each study were exposed and covered with gauze soaked in heated saline solution to keep moist until biopsy collection. Once \textit{in vivo} skeletal muscle biopsies for each study were taken from the right leg (see below), animals were humanely euthanized by excision of the heart, and the left leg was amputated for ischemic studies (see 2.3.c). Post-mortem verification of 2 ovaries in intact females and absence of ovaries in OVX females was confirmed by visual inspection. The uterus
of each female was excised, weighed (uw) and a uterus to body weight ratio (uw:bw) was calculated (refer to Appendix 2).

2.3.b. Skeletal Muscle Biopsies

All skeletal muscle biopsies were taken using an *in situ* freeze-clamp technique (Belanger *et al.*, 1992) and stored at -80°C until analysis. Skeletal muscles used for each study are as indicated: STUDY1: 3 primarily fast-twitch muscles *gracilis* (GRAC), *semitendinosus* (SMT), and *extensor digitorum longus* (EDL) muscles (Figure 2.5); STUDIES 2 & 3: predominately slow-twitch *soleus* (SOL) and predominately fast-twitch *extensor digitorum longus* (EDL) muscles (Figure 2.6). Muscles from the right leg were used to take *in vivo* biopsies and muscles from the left leg were used for the ischemic biopsy protocol. Pilot data confirmed that *in vivo* biopsies showed no differences in skeletal muscle metabolism between left and right legs.

![Study 1: Muscle Biopsies](image)

*Figure 2.5.* Muscles and metabolic variables measured in Study 1.
Figure 2.6. Muscles and metabolic variables measured in Studies 2 & 3.
2.3.c. Skeletal Muscle Ischemia

The left leg used for the ischemia experiments of Studies 2 and 3 was amputated and wrapped in moist gauze. A temperature probe was inserted into the upper thigh muscles to continually measure muscle temperature. This was maintained at 37°C by placing into a specialized warm chamber that kept the muscle temperature between 36.5-37.5°C. After 3 hours of ischemia, the SOL and EDL were removed and flash frozen in liquid nitrogen (Belanger et al., 1992) and stored at -80°C until analysis.

2.4. Biochemical Analyses

2.4.a. Enzymatic Analyses

For Study 1, all muscle biopsies were analyzed for the maximal activities of creatine kinase (CK), glycogen phosphorylase (GP), phosphofructokinase (PFK), lactate dehydrogenase (LDH), citrate synthase (CS), succinate dehydrogenase (SDH), beta-hydroxyacyl CoA dehydrogenase (βHADH), and cytochrome C oxidase (COX) using techniques previously described by Chi et al. (1983) and MacDougall et al. (1998). The enzymatic analyses were performed by the laboratory of Dr. Howard Green, University of Waterloo. Tissue homogenates were prepared as per Chi et al. (1983); 5 to 10 mg wet of muscle were homogenized in 50 volumes of 50% glycerol which contained 20 mM sodium phosphate buffer, pH 7.4, 5 mM β-mercaptoethanol, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% bovine serum albumin (BSA). These homogenates were used for the following enzymatic analysis as described below. Protein content was measured in homogenates using a Lowry protein assay as previously described (Chi et al., 1986).

**CK.** CK activity was determined by measuring the amount of NADPH produced when 1 µl of
diluted (1:3000) homogenate (0.3µg muscle) reacted with Reagent 1. Reagent 1 contained 100 mM imidazole-acetate, 25 mM creatine phosphate, 1 mM ADP, 10 mM Mg-acetate, 3 mM glucose, 20 mM AMP, 5 mM dithiothreitol, 0.02% BSA, 20 µg/ml yeast hexokinase, pH 6.1. After an hour, the reaction was stopped with 1N HCl (Figure 2.7.a. lines 1 & 2). Reagent 2 was added which contained 50 mM of Tris-HCl, 50µM NADP, 1 mM EDTA, 1 U/ml glucose-6-dehydrogenase, 0.35 U/ml phosphoglucoisomerase (added since some of the glucose-6-phosphate is converted to fructose-6-phosphate, and this enzyme reverts it back to glucose-6-phosphate). After 20 minutes, the amount of NADPH produced was measured flurometrically (Figure 2.7.a. line 3).

**PFK.** PFK activity was determined by measuring the amount of NAD\(^+\) produced using 10 µl of diluted (1:15,000) homogenate (0.66µg muscle). The homogenate was diluted with a reagent consisting of 50 mM Tris-HCl, 10 mM K\(_2\)HPO\(_4\), 5 mM mercaptoethanol, 0.5 mM EDTA, 0.2% BSA, pH 8.0. This was added to Reagent 1 which contained 50 mM Tris-HCl, 1 mM ATP, 1 mM fructose-6-phosphate, 10 mM K\(_2\)HPO\(_4\), 2 mM MgCl\(_2\), 200 µM NADH, 1 mM β-
mercaptoethanol, 0.05% BSA, 0.09U/ml aldolase, 1.5 U/ml glycerol-3-phosphate, 4.5 U/ml triosphosphate isomerase, pH 8.1 (Figure 2.7.b. lines 1-4). After an hour, the reaction was stopped with 0.75N HCl, and 1 ml of 6N NaOH was added and kept at 60°C for 20 minutes. The amount of NAD⁺ produced was measured flurometrically (Figure 2.7.b. line 4).

GP. GP activity was determined by measuring the amount of NADPH produced when 5 µl of diluted (1:1000) homogenate (5µg muscle) reacted with Reagent 1. Reagent 1 contained 50mM imidazole, pH 7.0, 50mM glycogen, 20mM K₂HPO₄, 0.5 mM MgCl₂, 1 mM 5'-AMP, 2 µM glucose-1,6-bisphosphate, 0.5 mM dithiotreitol, 0.25% BSA, 0.4 U/ml phosphoglucomutase (Figure 2.7.c. lines 1 & 2). After an hour, the reaction was stopped with 0.5N HCl. Reagent 2 was added which contained 50 mM of Tris-HCl, 50µM NADP, 1 mM EDTA, 1 U/ml glucose-6-dehydrogenase, 0.35 U/ml phosphoglucoisomerase (added since some of the glucose-6-phosphate is converted to fructose-6-phosphate, and this enzyme reverts it back to glucose-6-phosphate). After 20 minutes, the amount of NADPH produced was measured flurometrically (Figure 2.7.c. line 3).
LDH. LDH activity was determined by measuring the amount of NAD$^+$ produced using 10 µl of diluted (1:12,500) homogenate (0.8µg muscle). Homogenate was diluted with a reagent consisting of 200 mM imidazole and 0.1% BSA, pH 7.0. This was added to Reagent 1 which contained 100 mM imidazole, 2 mM Na-pyruvate, 300 µM NADH, 0.05% BSA, pH 7.0. (Figure 7.6.d). After an hour, the reaction was stopped with 0.5N HCl, and 1 ml of 6N NaOH was added and kept at 60°C for 20 minutes. The amount of NAD$^+$ produced was measured flurometrically (Figure 2.7.d.).

**Figure 2.7.c.** Metabolic reactions for glycogen phosphorylase analyses.

![Figure 2.7.c.](image)

**Figure 2.7.d.** Metabolic reaction for lactate dehydrogenase analyses.

![Figure 2.7.d.](image)
**CS.** CS was determined by measuring the amount of NAD$^+$ produced using 10 µl of diluted (1:2,500) homogenate (4 µg muscle). This was added to Reagent 1 which contained 50 mM Tris-HCl, 0.4 mM acetyl-CoA, 0.5 mM oxaloacetate, 0.25 % BSA, pH 8.1. After an hour, the reaction was stopped with 0.5N HCl, and heated at 95°C for 5 minutes to destroy excess oxaloacetate. Reagent 2 was added which contained 50 mM Tris-HCl, 100 µM ZnCl$_2$, 0.01% BSA, 30 µM NADH, 0.003 U/ml citrate lyase, 3 U/ml malate dehydrogenase, pH 7.5. After 20 minutes, reaction was stopped with 6N NaOH and kept at 60°C for 20 minutes. The amount of NAD$^+$ produced was measured flurometrically (Figure 2.7.e.).

![Figure 2.7.e. Metabolic reactions for citrate synthase analyses.](image)

**SDH.** SDH was determined by measuring the amount of NADH produced using 5 µl of diluted (1:250) homogenate (20 µg muscle). This was added to Reagent 1 which contained 50 mM imidazole-HCl, pH 7.4, 100 mM succinate, 10 mM K$_3$Fe(CN)$_6$, and 0.02% serum albumin. After an hour, the reaction was stopped with 1 N NaOH, and heated at 60°C for 20 minutes. Reagent 2 was added which contained 50 mM 2-amino-2-methyl-1,3-propanediol, pH 8.8, 200 µM NAD$^+$, 10 mM glutamate, 5 µg/ml fumarase, 2 µgm/ml glutamate-oxaloacetate transaminase, and 5 µg
of MDH. After 30 minutes, the amount of NADH produced was measured flurometrically (Figure 2.7.f.).

βHAD. βHAD was determined by measuring the amount of NAD$^+$ produced using 5 µl of diluted (1:7,500) homogenate (0.7 µg muscle). This was added to Reagent 1 which contained 150 mM imidazole-HCl, 200 µM acetoacetyl-CoA, 100 µM NADH, 1 mM EDTA, 0.05% BSA, pH 6.0. After an hour, the reaction was stopped with 0.5N HCl, and 1 ml of 6N NaOH was added and kept at 60°C for 20 minutes. The amount of NAD$^+$ produced was measured flurometrically (Figure 2.7.g.).

![Figure 2.7.f. Metabolic reactions for succinate dehydrogenase analyses.](image)

![Figure 2.7.g. Metabolic reactions for 3-beta hydroxyacyl dehydrogenase analyses.](image)
Cytochrome C Oxidase (COX). COX was determined by calculating the disappearance of reduced cytochrome C absorbance using a spectrofluorometric assay using 10 µl diluted (1:500) homogenate (20 µg) of muscle. Homogenate was diluted with 10 mM potassium phosphate buffer which contained 1mM of reduced cytochrome C. The decrease in absorbance at 550nm was recorded on a spectrofluorometer for 3 minutes.

2.4.b. Glycogen, Lactate, ATP, CP Analyses

All muscle biopsies were freeze-dried and cleaned of visible blood and connective tissue. To analyze samples for lactate, ATP and CP, a 3-5mg portion of each muscle was extracted in 600 µL of 0.5 M perchloric acid (PCA; EM Science, PX0396A-13) for 10 minutes in a cold ethanol bath of 0°C. Samples were centrifuged at 15000g for 10 minutes at 0°C (Centrifuge Eppendorf model 5402, Brinkmann Instruments Inc., Rexdale Ont.) and a 540 µL aliquot of extract was removed. Samples were neutralized with 135 µL of 2.3 M potassium bicarbonate (P-4913, Sigma Aldrich), centrifuged at 15000g for 10 minutes at 0°C, and the supernatant was stored at -80°C. Using fluorometric procedures (Passoneau & Lowry, 1993), all PCA extracted samples were analyzed for lactate, ATP and CP.

To analyze samples for glycogen, a 1-2 mg portion of each muscle was extracted in 1 ml of 1.0 M HCl for 1 hour at 99°C and then neutralized with 1.0 M of NaOH. The extract was filtered and analyzed using fluorometric procedures (Passoneau & Lowry, 1993).

2.4.c. Plasma 17β-Estradiol Analyses

Serum samples were prepared for radioimmunoassay (RIA) analyses by extracting them in diethyl ether to isolate 17β-estradiol from potentially interfering substances found in rat plasma. Briefly, 1.0 mL of diethyl ether was added to 200 µL of serum in a glass test tube, mixed
and then frozen in a dry ice bath. The organic phase was decanted into another glass test tube and the procedure was repeated with a second 1.0 mL of diethyl ether. The pooled organic phases were evaporated overnight under a fume hood and the extract was reconstituted with 200 µL of zero calibrator A (Coat-A-Count Estradiol, InterMedico, CA). Each serum sample was assessed for 17β-estradiol using a RIA kit (Coat-A-Count Estradiol, InterMedico, CA). Plasma estradiol levels were calculated in each female and summarized per Study in Appendix 3.

2.5. Mathematical & Statistical Analyses

All data were expressed as mean ± standard deviation (SD). The statistical analysis for each experimental study is described below. Significance was defined as $p \leq 0.05$. For Studies 2 and 3, the ischemic data were further analyzed mathematically as detailed in 2.5.a and 2.5.b.

2.5.a. Definition of Net Change in Metabolic Variables

To determine the change of the metabolic variables (X) during the ischemic interval, the differences between \textit{in vivo} values and ischemic values were calculated as follows:

\[
\text{Net X consumption (or accumulation) during ischemia} = X_{(3\text{ hours ischemia})} - X_{(in\text{ vivo})}
\]

The net amount of glycogen, CP, and ATP consumed and the net amount of lactate accumulated during the 3 hour ischemic period was determined. Note that when the net amount of glycogen, CP, and ATP was calculated, a negative value was generated which is reflective of the direction in change of these parameters, i.e. the number is negative because the metabolic
variable decreased during ischemia. Also, because the decrease in these measured variables is a result of that variable being consumed to sustain metabolic needs during ischemia, the term net consumption is used. Likewise, because lactate increased during ischemia, the term net accumulation is used to identify this process.

2.5.b. Definition of Net Change in Metabolic Variables Expressed as a Percentage of In Vivo Values

To correct for differences in the in vivo values, the percent glycogen, CP, and ATP consumption and the percent lactate accumulation were calculated as follows:

\[
\% \text{X consumed (or accumulated) during ischemia} = \frac{X_{(3 \text{ hours ischemia})} - X_{(in \text{ vivo})}}{X_{(in \text{ vivo})}} \times 100\
\]

2.5.c. Study 1: The data were statistically analyzed in 2 parts (i & ii) as follows:

i. To determine the role of estradiol on enzyme activity, the data were analyzed by comparing intact, OVX, and ET females using a one-way ANOVA followed by a Bonferonni post-hoc test within each specific fast-twitch muscle (GRAC, SMT, EDL). For data where the equal variance test failed, the data were analyzed using a Kruskal-Wallis one-way ANOVA on Ranks followed by a Dunn's test (SigmaStat, San Jose, CA).

ii. To determine the role of differing proportions of the fast-twitch fibers within fast-twitch skeletal muscles on enzyme activity in each experimental group, the data were analyzed by comparing GRAC, SMT, and EDL muscles using a one-way ANOVA followed by a
Bonferonni *post-hoc* test within each experimental group. For data where the equal variance test failed, the data were analyzed using a Kruskal-Wallis one-way ANOVA on Ranks followed by a Dunn's test (SigmaStat, San Jose, CA).

2.5.d. **Study 2**: The data were statistically analyzed as follows:

(i) **Effect of Estradiol and Progesterone**

To examine the effects of experimental treatment groups (OVX, IE, P, IE+P) within each specific muscle (EDL and SOL), the metabolic measurements (CP, glycogen, lactate, ATP) were analyzed using a one-way ANOVA followed by a Bonferroni *post-hoc* comparison for each of the following:

- *in vivo* metabolism
- 3 hour ischemic metabolism
- net change in metabolic parameter during ischemia
- percent change in metabolic parameter during ischemia

For data where the equal variance test failed, the data were analyzed using a Kruskal-Wallis one-way ANOVA on Ranks followed by a Dunn's test (SigmaStat, San Jose, CA).

(ii) **Effect of Skeletal Muscle Type (fast- versus slow-twitch)**

To examine the specific role of fast- and slow-twitch muscles (EDL vs SOL) within each experimental treatment group (OVX, IE, P, IE+P), the metabolic measurements (CP, glycogen,
lactate, ATP) were analyzed using a Student's t-test for each of the following:

- *in vivo* metabolism
- 3 hour ischemic metabolism
- net change in metabolic parameter during ischemia
- percent change in metabolic parameter during ischemia

2.5.e. Study 3: The data were statistically analyzed as follows:

(i) Effect of ER Activation

To examine the effects of experimental treatment groups (OVX, ICI, RLX) within each specific muscle (EDL and SOL), the metabolic measurements (CP, glycogen, lactate, ATP) were analyzed using a one-way ANOVA followed by a Bonferroni post-hoc comparison for each of the following:

- *in vivo* metabolism
- 3 hour ischemic metabolism
- net change in metabolic parameter during ischemia
- percent change in metabolic parameter during ischemia

For data where the equal variance test failed, the data were analyzed using a Kruskal-Wallis one-way ANOVA on Ranks followed by a Dunn's test (SigmaStat, San Jose, CA).

(ii) Effect of Skeletal Muscle Type (fast- versus slow-twitch)

To examine the specific role of fast- and slow-twitch muscles (EDL vs SOL) within each
experimental treatment group (OVX, ICI, RLX), the metabolic measurements (CP, glycogen, lactate, ATP) were analyzed using a Student's t-test for each of the following:

- *in vivo* metabolism
- 3 hour ischemic metabolism
- net change in metabolic parameter during ischemia
- percent change in metabolic parameter during ischemia
CHAPTER 3

RESULTS
3.1 STUDY 1: Regulation of metabolic enzymes by estradiol in fast-twitch skeletal muscles

The results will first identify the effect of estradiol on the activity of each enzyme in the 3 fast-twitch skeletal muscles *gracilis* (GRAC), *semitendinosus* (SMT), and *extensor digitorum longus* (EDL). As well, the effects of differing proportions of fast-twitch fibers in fast-twitch muscles on enzyme activity in the 3 experimental groups, gonadally intact (INT), ovariectomized (OVX) and OVX + estradiol-treated (ET) females, will be identified. The enzyme activity data in all three experimental groups and muscles are summarized in Table 3.1.1. Where statistically significant effects were observed, data were graphed to better illustrate the results. Specifically, GP, PFK, and LDH data were graphed in Figures 3.1.1, 3.1.2, and 3.1.3, respectively.

3.1.a. CREATINE KINASE (CK)

3.1.a.(i). The effect of estradiol on CK activity in the GRAC, SMT, EDL muscles

In the GRAC, CK activity of ET females was not statistically different when compared to OVX. However, OVX had 33% less CK activity (p=0.004) compared to INT females (Table 3.1.1) and CK activity in ET was not statistically different compared to INT. In both the SMT and EDL muscles, CK activity was not statistically different among INT, OVX, and ET females.

3.1.a.(ii). The effect of differing fast-twitch muscles on CK activity in each experimental group

In either INT, OVX, and ET females, CK activity was not statistically different among GRAC, SMT, and EDL muscles.
Table 3.1.1. Enzyme activity of CK, GP, PFK, LDH, CS, SDH, βHAD, and COX (expressed as mol/kg protein/hr) in the GRAC, SMT, and EDL muscles of female Sprague-Dawley rats who were either sham-operated (INT, n=5), ovariectomized (OVX, n=6), or estradiol treated (ET, n=6). All values expressed as mean ± SD. a p ≤ 0.05, INT vs OVX within same muscle; b p ≤ 0.05, OVX vs ET within the same muscle; c p ≤ 0.05, INT vs ET within same muscle; * p ≤ 0.05 vs GRAC within the same experimental group.

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<td>14.7 ± 2.5³</td>
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<td>12.3 ± 2.2³</td>
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<td>19.7 ± 1.7²*</td>
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<tr>
<td>SMT</td>
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<td>1.8 ± 0.9</td>
<td>1.2 ± 1.3</td>
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<td>1.1 ± 0.3</td>
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<td>Electron Transport Chain</td>
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<td>61.9 ± 14.7</td>
<td>46.9 ± 16.6</td>
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</table>
3.1.b. PHOSPHOFRUCTOKINASE (PFK)

3.1.b.(i). The effect of estradiol on PFK activity in the GRAC, SMT, EDL muscles

In the SMT, PFK activity was 23% lower (p=0.012) in ET females compared to OVX females (Figure 3.1.1). Although PFK activity in the SMT of OVX females appeared greater compared to INT females, this did not achieve statistical significance (p=0.063). PFK activity in the SMT was not statistically different between ET and INT females. In the GRAC and EDL muscles, PFK activity was not statistically different among INT, OVX, and ET females.

3.1.b.(ii). The effect of differing fast-twitch muscles on PFK activity in each experimental group

In OVX females, there were muscle specific differences in PFK activity (Figure 3.1.1). Specifically, compared to the GRAC, PFK activity was 36% greater (p=0.008) in the SMT and 30% greater (p=0.047) in the EDL; however, PFK activity was not statistically different between the SMT and EDL muscles. In both INT and ET females, PFK activity was not significantly different among the GRAC, SMT, and EDL muscles.
**Figure 3.1.1.** PFK activity of the GRAC, SMT, EDL muscles. Intact (INT, n=5), ovariectomized (OVX, n=6), estradiol-treated (ET, n=6) female rats. GRAC; SMT; EDL. Values are expressed as means ± SD. °p ≤ 0.05 vs OVX within same muscle; *p ≤ 0.05 vs GRAC within same experimental group.
3.1.c. GLYCOGEN PHOSPHORYLASE (GP)

3.1.c.(i). The effect of estradiol on GP activity in the GRAC, SMT, EDL muscles

In the GRAC, GP activity was not statistically (p=0.063) different among INT, OVX, and ET females (Figure 3.1.2). In the SMT, GP activity was 29% lower (p=0.013) in ET females compared to OVX females (Figure 3.1.2) and 38% greater (p=0.003) in OVX females compared to INT females, with no statistical difference in GP activity between INT and ET females. As observed in the SMT, GP activity had similar results to OVX and ET in the EDL. GP activity in the EDL was 31% lower (p=0.003) in ET females compared to OVX females and 27% greater (p≤0.001) in OVX females compared to INT females, with no statistical difference in GP activity between INT and ET females.

3.1.c.(ii). The effect of differing fast-twitch muscles on GP activity in each experimental group

In INT females, GP activity was not significantly different among GRAC, SMT, and EDL muscles. In OVX females, differences in GP activity were observed (Figure 3.1.2). GP activity of OVX females was 28% lower (p≤0.05) in the GRAC compared to the EDL. GP activity of OVX females was not statistically different between SMT and EDL muscles and although GP activity had a lower mean value in the GRAC compared to the EDL, this was not statistically different. In ET females, GP activity was not significantly different among the GRAC, SMT, and EDL muscles (p=0.091).
Figure 3.1.2. GP activity of the GRAC, SMT, EDL muscles. Intact (INT, n=5), ovariectomized (OVX, n=6), estradiol-treated (ET, n=6) female rats. GRAC; SMT; EDL. Values are expressed as means ± SD. \( ^{a} p \leq 0.05 \) vs INT within same muscle; \( ^{b} p \leq 0.05 \) vs OVX within same muscle; \( ^{*} p \leq 0.05 \), vs GRAC within same experimental group.
3.1.d. LACTATE DEHYDROGENASE (LDH)

3.1.d.(i). The effect of estradiol on LDH activity in the GRAC, SMT, EDL muscles

In the GRAC, LDH activity was 26% lower (p=0.002) in OVX compared to INT females and giving estradiol did not change this as LDH activity was 31% lower (p=0.008) in ET compared to INT females (Figure 3.1.3). Additionally, LDH activity in ET females was not significantly different compared to OVX females in the GRAC. In the SMT, LDH activity was 20% lower in ET females compared to OVX females, but this was a trend to significance (p=0.058). In the SMT, LDH activity was not statistically different between OVX and INT females or between INT and ET females. In the EDL, LDH activity was not significantly different among INT, OVX, and ET females.

3.1.d.(ii). The effect of differing fast-twitch muscles on LDH activity in each experimental group

In OVX females, LDH activity was 26% lower (p≤0.05) in the GRAC compared to the SMT, and LDH activity was not statistically different between SMT and EDL. In OVX females, despite LDH activity having the lowest mean value in the GRAC, it was not statistically different compared to the EDL (p=0.153). In INT and ET females, LDH activity was not statistically different among the GRAC, SMT, EDL muscles.
Figure 3.1.3. LDH activity of the GRAC, SMT, EDL muscles. Intact (INT, n=5), ovariectomized (OVX, n=6), estradiol-treated (ET, n=6) female rats. GRAC; SMT; EDL. Values are expressed as means ± SD. a p≤0.05 vs INT within same muscle; * p≤0.05, vs GRAC within same experimental group.
3.1.e. CITRATE SYNTHASE (CS)

3.1.e.(i). The effect of estradiol on CS activity in the GRAC, SMT, EDL muscles

CS activity was not statistically different among INT, OVX, and ET females in each of the muscles studied (GRAC, SMT, EDL) (Table 3.1.1).

3.1.e.(ii). The effect of differing fast-twitch muscles on CS activity in each experimental group

In OVX females, CS activity was not statistically (p=0.055) different among the GRAC, SMT, and EDL muscles. In INT and ET females, CS activity was not statistically different among the GRAC, SMT, and EDL muscles.

3.1.f. SUCCINATE DEHYDROGENASE (SDH)

3.1.f.(i). The effect of estradiol on SDH activity in the GRAC, SMT, EDL muscles

In the GRAC, although SDH activity was not statistically different between ET and OVX females, ET females had 33% less (p=0.014) SDH activity compared to INT females, with no significant difference in SDH activity between INT and OVX females (Table 3.1.1). In the SMT and EDL muscles, SDH activity was not statistically different among INT, OVX, and ET females.

3.1.f.(ii). The effect of differing fast-twitch muscles on SDH activity in each experimental group

In INT females, SDH activity was not statistically different among the GRAC, SMT, and
EDL muscles. In OVX females, SDH activity was 35% lower in the SMT compared to GRAC and EDL, but this was a trend to significance (p=0.051). In ET females, SDH activity was not statistically (p=0.061) different among the GRAC, SMT, and EDL muscles.

3.1.g BETA HYDROXYACYL DEHYDROGENASE (βHAD)

3.1.g.(i). The effect of estradiol on βHAD activity in the GRAC, SMT, EDL muscles

βHAD activity was not statistically different among INT, OVX, and ET females in each of the muscles studied (GRAC, SMT, EDL) (Table 3.1.1).

3.1.g.(ii). The effect of differing fast-twitch muscles on βHAD activity in each experimental group

In INT, OVX, and ET females, βHAD activity was not statistically different among the GRAC, SMT, and EDL muscles.

3.1.h. Cytochrome C Oxidase (COX)

3.1.h.(i). The effect of estradiol on COX activity in the GRAC, SMT, EDL muscles

In the GRAC, COX activity was not statistically different between OVX and ET females (Table 3.1.1). It should be noted that COX activity was 27% lower (p=0.051) in ET females compared to OVX females, but this was a trend to significance. In SMT and EDL muscles, COX activity was not statistically different among INT, OVX, and ET females.
3.1.h.(ii). The effect of differing fast-twitch muscles on COX activity in each experimental group

In INT, OVX, and ET females, COX activity was not statistically different among the GRAC, SMT, and EDL muscles.
SUMMARY OF RESULTS FOR STUDY 1

The results of Study 1 identified important skeletal muscle differences in their response to estradiol. The findings emphasized that even among fast-twitch muscles, estradiol affected muscles with a greater proportion of total Type II fibers, highlighting that not all fast-twitch muscles respond equally to estradiol. From a metabolic perspective, estradiol’s effects were prominent on metabolic pathways that fast-twitch muscles are adapted for, which is rapid energy production under conditions of high functional demand. Thus, estradiol regulated glycogenolysis (GP) and glycolysis (PFK) which provide substrates needed for the above scenarios. Estradiol decreased the activity of GP, which appeared to be more significant in muscles with a greater proportion of total Type II fibers (SMT, EDL). Estradiol also decreased PFK activity; however, this occurred only in the SMT, reaffirming that not all fast-twitch muscles respond equally to estradiol. These data also illustrated that the activity of various enzymes that participate in the Citric Acid Cycle (CS, SDH), beta-oxidation of fatty acids (βHAD), and mitochondrial oxidative phosphorylation (COX) were not altered by OVX or estradiol replacement in any of the three fast-twitch muscles studied. This is most likely attributed to the finding that these muscles had low activity levels of most of the enzymes belonging to oxidative metabolism except for COX.
3.2: STUDY 2: The role of intermittent-estradiol and progesterone in skeletal muscle - 
effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

In this section, the findings from the 4 experimental groups, OVX, OVX+IE (IE), OVX+P (P), and OVX given a combination of both IE+P (IE+P) are presented. The effect of these experimental groups was examined in two different skeletal muscle types, fast-twitch EDL and the slow-twitch SOL muscles. These muscles were selected because of their contrasting metabolic properties (i.e. fast-twitch muscles have greater glycolytic potential versus slow-twitch muscles which have lower glycolytic potential). In vivo muscle biopsies were taken (from the right leg) to determine what effect the experimental groups had on 'resting' muscle. An experiment was also performed to determine the metabolic response of these muscles to three hours of ischemia in all experimental groups (ischemia protocol performed on left leg). The metabolic data measured were glycogen, CP, ATP, and lactate as these variables represent metabolic pathways important to ischemic metabolism in skeletal muscle. Where statistically significant effects were observed, data were graphed to better illustrate the results.

3.2.a.(i). The effect of female sex hormones on in vivo CP levels in fast- and slow-twitch muscles.

In the fast-twitch EDL, in vivo CP levels were not statistically different between IE and OVX females (Figure 3.2.2). However, in vivo CP levels were 17% lower (p=0.033) in P females compared to IE females (Figure 3.2.2), while in vivo CP levels were similar between P and OVX females. When both hormones were given, IE+P females had 19% (p=0.034) and 24% (p=0.002) lower in vivo CP levels compared to OVX and IE females, respectively.

In the slow-twitch SOL, in vivo CP levels were not statistically different between IE and
OVX females (Figure 3.2.2). In P females, *in vivo* CP levels were 20% lower compared to OVX females, but this was a trend to significance (p=0.079). When both hormones were given, IE+P females had 25-27% lower *in vivo* CP levels compared to both OVX (p=0.017) and IE (p=0.033) females (Figure 3.2.2).

3.2.a.(ii). The effect of fast- versus slow-twitch muscles on *in vivo* CP levels within each experimental group.

*In vivo* CP levels of the SOL were significantly lower compared to EDL muscles within each experimental group. Specifically, compared to the EDL, *in vivo* CP levels in the SOL were 38% lower (p≤0.001) in OVX, 43% lower (p=0.002) in IE, 44% lower (p≤0.001) in P, and 44% lower (p≤0.001) in IE+P females (Figure 3.2.2).
Figure 3.2.1. *In Vivo* CP levels. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. *a* p ≤ 0.05 vs OVX within same muscle, *b* p ≤ 0.05 vs IE within same muscle, *p* ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.2.b.(i). The effect of female sex hormones on \textit{in vivo} glycogen levels in fast- and slow-twitch muscles.

In the fast-twitch EDL, \textit{in vivo} glycogen levels were not statistically different among OVX, IE, and P females (Figure 3.2.1). However, \textit{in vivo} glycogen levels of IE+P females were 25\% lower (p=0.006) compared to OVX and 22\% lower (p=0.026) compared to IE females (Figure 3.2.1).

In the slow-twitch SOL, \textit{in vivo} glycogen levels were not statistically different between IE and OVX females (Figure 3.2.1). However, P females had significantly lower \textit{in vivo} glycogen levels by 33\% compared to both OVX (p=0.005) and IE (p=0.006) females (Figure 3.2.1). When both hormones were given, IE+P females had 25\% lower \textit{in vivo} glycogen levels compared to both OVX (p=0.042) and IE (p=0.05) females, while no differences were observed between IE+P and P females (Figure 3.2.1).

3.2.b.(ii). The effect of fast- versus slow-twitch muscles on \textit{in vivo} glycogen levels within each experimental group.

\textit{In vivo} glycogen levels were not statistically different between SOL and EDL muscles in either OVX, IE, and IE+P females (Figure 3.2.1). However, in P females, \textit{in vivo} glycogen levels in the SOL muscle was approximately 30\% (p=0.013) lower compared to the EDL (Figure 3.2.1).
Figure 3.2.2. *In vivo* glycogen levels. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs IE within same muscle, * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.2.c.(i). The effect of female sex hormones on in vivo ATP levels in fast- and slow-twitch muscles.

In the fast-twitch EDL, in vivo ATP levels were not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.3). However, IE females had 18% greater in vivo ATP levels compared to P females, but this was a trend to significance (p=0.086) (Figure 3.2.3).

In the slow-twitch SOL, in vivo ATP levels were not significantly different between IE and OVX females and P and OVX females (Figure 3.2.3). However, IE females had 23% (p=0.021) greater in vivo ATP levels compared to P females (Figure 3.2.3). In vivo ATP levels of IE+P females were not statistically different compared to other experimental groups.

3.2.c.(ii). The effect of fast- versus slow-twitch muscles on in vivo ATP levels within each experimental group.

In vivo ATP levels of the SOL were significantly lower compared to EDL muscles within each experimental group. Specifically compared to the EDL, in vivo ATP levels in the SOL were 35% lower (p≤0.001) in OVX, 36% lower (p=0.002) in IE, 39% lower (p≤0.001) in P, and 36% lower (P≤0.001) in IE+P females (Figure 3.2.3).
Figure 3.2.3. \textit{In vivo} ATP levels. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. \( b \ p \leq 0.05 \) vs IE within same muscle, \( * \ p \leq 0.05 \) vs EDL within same treatment group. \( n=6 \) in all groups.
3.2.d.(i). The effect of female sex hormones on *in vivo* lactate levels in fast- and slow-twitch muscles.

In both the fast-twitch EDL and slow-twitch SOL, *in vivo* lactate levels were not statistically different among OVX, IE, P, and IE+P females (Table 3.2.4).

3.2.d.(ii). The effect of fast- versus slow-twitch muscles on *in vivo* lactate levels within each experimental group.

*In vivo* lactate levels were low in both the SOL and EDL. *In vivo* lactate levels were not statistically different between SOL and EDL muscles in OVX, IE, P, and IE+P females (Table 3.2.4).
ISCHEMIC DATA

3.2.e.(i). The effect of female sex hormones on ischemic CP levels in fast- and slow-twitch muscles.

CP levels were markedly reduced after 3 hours of ischemia in all experimental groups, and the following describes the effect of estradiol and progesterone on this response (Table 3.2.2).

In the fast-twitch EDL, ischemic CP levels were not statistically different among OVX, IE, P, and IE+P females. The net CP consumption during ischemia was not statistically different among OVX, IE, and P females (Figure 3.2.6). Interestingly, the net CP consumption of IE+P females was 21% lower (p=0.022) compared to OVX and 25% lower (p=0.003) compared to IE females (Figure 3.2.6). The percent CP consumption was not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.6).

In the slow-twitch SOL, ischemic CP levels, the net CP consumption during ischemia, and the percent CP consumption during ischemia were not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.6).

3.2.e.(ii). The effect of fast- versus slow-twitch muscles on ischemic CP levels within each experimental group.

Ischemic CP levels were not statistically different between SOL and EDL in all experimental groups. The net CP consumption during ischemia was significantly lower in the SOL compared to the EDL in OVX, IE, P, and IE+P females. Specifically compared to EDL, the net SOL CP consumption was 39% lower (p≤0.001) in OVX, 46% lower (p=0.004) in IE, 47% lower (p≤0.001) in P, and 42% lower (p≤0.001) in IE+P females (Figure 3.2.6). The percent CP
consumption was not statistically different between SOL and EDL muscles in OVX, IE, and IE+P females. In P females, the percent SOL CP consumption was 6% lower (p=0.029) compared to the EDL.
Figure 3.2.4. (i) Net CP consumption (ii) % CP consumed during ischemia. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs IE within same muscle, * p ≤ 0.05 vs EDL within same treatment group. n=6 in all groups.
### Table 3.2.1

<table>
<thead>
<tr>
<th>CP</th>
<th>Muscle</th>
<th>Experimental Group</th>
<th>O VX</th>
<th>IE</th>
<th>P</th>
<th>IE+P</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>EDL</td>
<td>96.3 ± 5.2</td>
<td>103.2 ± 17.9</td>
<td>84.6 ± 5.2 (^b)</td>
<td>77.8 ± 7.4 (^{a,b})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>60.2 ± 5.3 (^*)</td>
<td>58.8 ± 12.4 (^*)</td>
<td>47.5 ± 6.3 (^{a,*})</td>
<td>43.6 ± 5.4 (^{a,b,*})</td>
<td></td>
</tr>
<tr>
<td>3 Hours Ischemia (µmol/g dw)</td>
<td>EDL</td>
<td>6.6 ± 5.8</td>
<td>8.8 ± 6.0</td>
<td>3.2 ± 2.5</td>
<td>6.6 ± 8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>5.2 ± 4.8</td>
<td>7.9 ± 4.7</td>
<td>5.0 ± 2.6</td>
<td>1.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Net Consumption (µmol/g dw)</td>
<td>EDL</td>
<td>-89.7 ± 9.9</td>
<td>-94.4 ± 14.6</td>
<td>-80.9 ± 6.0</td>
<td>-71.2 ± 4.3 (^{a,b})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-55.0 ± 8.6 (^*)</td>
<td>-51.0 ± 15.9 (^*)</td>
<td>-42.6 ± 5.3 (^*)</td>
<td>-41.5 ± 6.2 (^*)</td>
<td></td>
</tr>
<tr>
<td>% CP consumed</td>
<td>EDL</td>
<td>-93.0 ± 6.4</td>
<td>-91.8 ± 5.2</td>
<td>-96.2 ± 3.0</td>
<td>-92.2 ± 9.5</td>
<td></td>
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<td></td>
<td>SOL</td>
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<td>-85.5 ± 8.2</td>
<td>-89.7 ± 4.8 (^*)</td>
<td>-94.9 ± 4.8</td>
<td></td>
</tr>
</tbody>
</table>

CP content (µmol/g dw) from *in vivo* and ischemic tissue. Placebo (OVX), intermittent-estradiol (IE), progesterone (P), or both IE and P (IE+P). Net CP consumption (calculated as *in vivo* CP minus 3 hour ischemic CP value), and % CP consumed (net CP consumption expressed as percentage of *in vivo* value). Values are all expressed as mean ± SD. \(^a\) p ≤ 0.05 vs OVX within same muscle, \(^b\) p ≤ 0.05 vs IE within same muscle, \(*\) p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.2.f.(i). The effect of female sex hormones on ischemic glycogen levels in fast- and slow-twitch muscles.

Glycogen levels were markedly reduced after 3 hours of ischemia in all experimental groups, and the following describes the effect of estradiol and progesterone on this response (Table 3.2.1).

In the fast-twitch EDL, ischemic glycogen levels were not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.4). Net glycogen consumption during ischemia was not statistically different among OVX, IE, and OVX females (Figure 3.2.5). Interestingly, the net glycogen consumption was 25% lower (p=0.005) in IE+P females compared to OVX females and 23% lower (p=0.013) compared to IE females (Figure 3.2.5). The percent glycogen consumption was not statistically different among OVX, IE, P, and IE+P females, with most females consuming over 94% of EDL glycogen stores during ischemia.

In the slow-twitch SOL, ischemic glycogen levels were not statistically different between IE and OVX females and P and OVX females (Figure 3.2.4); however, ischemic glycogen levels of P females were 67% lower (p=0.010) compared to IE females. Ischemic glycogen levels of IE+P were 52% lower compared to IE females, but this was a trend to significance (p=0.058). Net glycogen consumption during ischemia was not statistically different between IE did and OVX females; however, P females had 30% lower (p=0.006) net glycogen consumption compared to OVX females (Figure 3.2.5). When given both hormones, IE+P females had 25% lower (p=0.023) net glycogen consumption compared to OVX females. The percent glycogen consumption of P females was 16% greater (p=0.050) compared to IE females (Figure 3.2.5) and no other differences were observed.
3.2.f.(ii). The effect of fast- versus slow-twitch muscles on ischemic glycogen levels within each experimental group.

In all of the experimental groups, with the exception of the P group, ischemic glycogen levels in the SOL were significantly greater compared to the EDL (Figure 3.2.4). Specifically compared to EDL, the ischemic glycogen levels in the SOL were 66% greater (p=0.026) in OVX, 83% greater (p=0.002) in IE, and 70% greater (p=0.009) in IE+P females (Figure 3.2.4). The net glycogen consumption during ischemia was not statistically different between SOL and EDL muscles in OVX and IE+P females. The net SOL glycogen consumption was 19% lower (p=0.040) in IE and 29% lower (p=0.001) in P females compared to the EDL (Figure 3.2.5). The percent glycogen consumption of the SOL was 12% lower (p=0.009) in OVX, 23% lower (p=0.002) in IE, and 13% lower (p=0.002) in IE+P females compared to EDL, and no muscle differences were observed in P females (Figure 3.2.5).
Figure 3.2.5. Glycogen levels at 3 hours of ischemia. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. \( b \ p \leq 0.05 \) vs IE within same muscle, \( * \ p \leq 0.05 \) vs EDL within same treatment group. \( n=6 \) in all groups.
Figure 3.2.6. (i) Net glycogen consumption (ii) % glycogen consumed during ischemia. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs IE within same muscle, * p ≤ 0.05 vs EDL within same treatment group. n=6 in all groups.
<table>
<thead>
<tr>
<th>GLYCOGEN</th>
<th>Muscle</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OVX</td>
</tr>
<tr>
<td>In Vivo (µmol glucosyl units/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>103.2 ± 11.6</td>
<td>99.0 ± 9.7</td>
</tr>
<tr>
<td>SOL</td>
<td>105.8 ± 14.5</td>
<td>105.1 ± 22.0</td>
</tr>
<tr>
<td>3 Hours Ischemia (µmol glucosyl units /g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>6.5 ± 2.3</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>SOL</td>
<td>18.9 ± 11.3</td>
<td>29.0 ± 11.6 *</td>
</tr>
<tr>
<td>Net Consumption (µmol glucosyl units /g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>-96.7 ± 9.9</td>
<td>-94.2 ± 8.7</td>
</tr>
<tr>
<td>SOL</td>
<td>-86.9 ± 9.5</td>
<td>-76.1 ± 16.6 *</td>
</tr>
<tr>
<td>% Glycogen Consumed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>-93.8 ± 1.6</td>
<td>-95.2 ± 0.7</td>
</tr>
<tr>
<td>SOL</td>
<td>-82.9 ± 9.0*</td>
<td>-72.8 ± 9.2 *</td>
</tr>
</tbody>
</table>

Table 3.2.2. Glycogen content (µmol glucosyl units/g dw) from in vivo and ischemic skeletal muscles. Placebo (OVX), intermittent-estradiol (IE), progesterone (P), or both IE and P (IE+P). Net glycogen consumption (calculated as in vivo glycogen minus 3 hour ischemic glycogen value), and % glycogen consumed (net glycogen consumption expressed as percentage of in vivo value). Values are all expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs IE within same muscle, * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.2.g.(i). The effect of female sex hormones on ischemic ATP levels in fast- and slow-twitch muscles.

ATP levels were markedly reduced after 3 hours of ischemia in all experimental groups, and the following describes the effect of estradiol and progesterone on this response (Table 3.2.3).

In the fast-twitch EDL, ischemic ATP levels, the net ATP consumption during ischemia, and the percent ATP consumption during ischemia were not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.7).

In the slow-twitch SOL, ischemic ATP levels were not statistically significant among OVX, IE, P, and IE+P females. The net ATP consumption during ischemia was not statistically significant between IE and OVX; however, the net ATP consumption in P females was 22% lower (p=0.015) compared to IE females (Figure 3.2.7). The net ATP consumption of IE+P females was similar to other experimental groups. The percent ATP consumption was not statistically different among OVX, IE, P, and IE+P females (Figure 3.2.7).

3.2.g.(ii). The effect of fast- versus slow-twitch muscles on ischemic ATP levels within each experimental group.

In OVX females, ischemic ATP levels of the SOL were 33% lower (p=0.041) compared to the EDL. Ischemic ATP levels were not statistically significant between SOL and EDL in IE, P, and IE+P females. The net ATP consumption during ischemia was significantly lower in the SOL compared to the EDL among OVX, IE, P, and IE+P females (Figure 3.2.7). Specifically compared to the EDL, the net SOL ATP consumption was 37% lower (p≤0.001) in OVX, 38% lower (p=0.002) in IE, 37% lower (p≤0.001) in P, and 33% lower (p≤0.001) in IE+P females.
(Figure 3.2.7). The percent ATP consumption was not statistically significant between SOL and EDL muscles in OVX, IE, and IE+P females. There was a small difference in the percent ATP consumption in P females which was 1% lower (p=0.049) in the SOL compared to the EDL.
Figure 3.2.7. (i) Net ATP consumption (ii) % ATP consumed during ischemia. OVX female Sprague-Dawley rats were given placebo (OVX), intermittent-estradiol treatment (IE), progesterone (P), or both IE and P (IE+P). EDL, SOL. All values expressed as mean ± SD. $^b$ p ≤ 0.05 vs IE within same muscle, * p ≤ 0.05 vs EDL within same treatment group. n=6 in all groups.
Table 3.2.3. ATP content (µmol/g dw) from *in vivo* and ischemic tissue. Placebo (OVX), intermittent-estradiol (IE), progesterone (P), or both IE and P (IE+P). Net ATP consumption (calculated as *in vivo* ATP minus 3 hour ischemic ATP value), and % ATP consumed (net ATP consumption expressed as percentage of *in vivo* value). Values are all expressed as mean ± SD.

<table>
<thead>
<tr>
<th>ATP</th>
<th>Muscle</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OVX</td>
</tr>
<tr>
<td><em>In Vivo</em> (µmol/g dw)</td>
<td>EDL</td>
<td>31.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>19.8 ± 1.1 *</td>
</tr>
<tr>
<td>3 Hours Ischemia</td>
<td>EDL</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>SOL</td>
<td>0.8 ± 0.06 *</td>
</tr>
<tr>
<td>Net Consumption</td>
<td>EDL</td>
<td>-30.2 ± 2.5</td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>SOL</td>
<td>-19.0 ± 1.0 *</td>
</tr>
<tr>
<td>% ATP Consumed</td>
<td>EDL</td>
<td>-96.1 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-96.0 ± 0.3</td>
</tr>
</tbody>
</table>

*p ≤ 0.05 vs IE within same muscle, *p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.2.h.(i). The effect of female sex hormones on ischemic lactate levels in fast- and slow-twitch muscles.

Lactate levels were markedly increased after 3 hours of ischemia in all experimental groups, and the following describes the effect of estradiol and progesterone on this response (Table 3.2.4).

In both the fast-twitch EDL and slow-twitch SOL, ischemic lactate levels, the net lactate accumulation during ischemia, and the percent lactate accumulation during ischemia were not statistically different among OVX, IE, P, and IE+P females.

3.2.h.(ii). The effect of fast- versus slow-twitch muscles on ischemic lactate levels within each experimental group.

Lactate levels after 3 hours of ischemia were not statistically different between SOL and EDL muscles in OVX, IE, and IE+P females; however in P females, SOL had 16% less (p=0.010) lactate compared to the EDL (Table 3.2.4). The net lactate accumulation during ischemia was not statistically different between SOL and EDL muscles in OVX, IE, and IE+P females; however in P females, SOL had 16% lower (p=0.012) values compared to the EDL. The percent lactate accumulation was not statistically different between SOL and EDL muscles in OVX, IE, P, and IE+P females.
### Table 3.2.4

Lactate content (µmol/g dw) from *in vivo* and ischemic tissue. Placebo (OVX), intermittent-estradiol (IE), progesterone (P), or both IE and P (IE+P). Net lactate accumulation (calculated as *in vivo* lactate minus 3 hour ischemic lactate value), and % lactate accumulated (net lactate accumulation expressed as percentage of *in vivo* value). Values are all expressed as mean ± SD. * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.

<table>
<thead>
<tr>
<th>LACTATE</th>
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<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OVX</td>
</tr>
<tr>
<td>In Vivo (µmol/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>6.9 ± 2.6</td>
<td>8.5 ± 4.1</td>
</tr>
<tr>
<td>SOL</td>
<td>5.6 ± 1.5</td>
<td>6.8 ± 3.1</td>
</tr>
<tr>
<td>3 Hours Ischemia (µmol/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>235.4 ± 29.7</td>
<td>230.0 ± 33.4</td>
</tr>
<tr>
<td>SOL</td>
<td>204.6 ± 21.8</td>
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<td>Net Accumulation (µmol/g dw)</td>
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<tr>
<td>EDL</td>
<td>+228.5 ± 27.5</td>
<td>+221.5 ± 34.1</td>
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<tr>
<td>SOL</td>
<td>+199.0 ± 22.2</td>
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<td>% Lactate Accumulated</td>
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<tr>
<td>SOL</td>
<td>+3783 ± 1188</td>
<td>+3595 ± 1774</td>
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</table>
Summary of Results for Study 2

In vivo:

Overall, progesterone decreased EDL glycogen levels in estradiol-primed females, but had no effect alone relative to OVX and IE females. In contrast, progesterone alone decreased SOL glycogen levels, which was not reversed when given IE. IE alone had no effect on glycogen levels compared to OVX females in either muscle. Progesterone alone decreased EDL CP levels, but compared to IE and not OVX females, and had no effect in the SOL. Interestingly, when IE and progesterone were given together, CP decreased in both muscles. These changes in glycogen and CP levels did not affect ATP in either muscle, and lactate levels were not affected by these hormones in either muscle.

Ischemia:

Progesterone decreased net EDL glycogen consumption in estradiol-primed females, but had no effect alone relative to OVX and IE females. Interestingly, the percent glycogen consumed by the EDL was similar between IE and progesterone, suggesting that the ability to break down glycogen was not impaired. Thus, the decreased net glycogen consumption due to progesterone in the EDL was attributed to lower in vivo glycogen levels. In contrast, progesterone alone reduced SOL net glycogen consumption relative to OVX females only, which was not reversed when given IE. Both progesterone alone and given in combination with estradiol resulted in similar percent glycogen consumption in SOL relative to OVX, suggesting that decreased net glycogen consumption in the SOL is attributed to reduced in vivo glycogen levels in this group. Additionally, IE decreased percent glycogen consumption in the SOL relative to progesterone alone, which was reversed in IE+P females. This suggests that estradiol
decreased the ability to break down glycogen in the SOL, since \textit{in vivo} glycogen levels in this group were unchanged.

Both IE+P decreased net EDL CP consumption, but neither estradiol nor progesterone alone had an effect relative to OVX. However, percent EDL CP consumption was not altered by IE+P, suggesting that decreased EDL CP consumption was attributed to lower \textit{in vivo} CP levels. In contrast, IE+P had no effect on SOL CP ischemic profiles.

Progesterone decreased SOL ATP consumption and percent ATP consumed relative to IE, but this was reversed when both IE+P were given. Despite the percent ATP consumption being statistically significant between IE and P, the biological relevance of such a minute difference is not relevant as this difference is extremely small (94.9\% vs 96.7\%, difference = 1.8\%).

Lactate production as a result of anaerobic glycolysis was not affected by either IE, progesterone or when given together in either muscles.

Thus, the biological effects of a low-dose estradiol pellet (IE) resulted in significant findings in glycogen and CP metabolism in skeletal muscle, especially when given in combination with progesterone. There were also muscle fiber type effects illustrated, reiterating the concept that skeletal muscle fiber types need to be considered when addressing the effects of hormones, such as estradiol and progesterone, on skeletal muscle metabolism.
3.3 STUDY 3: The role of ER in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

In this section, the findings from the 3 experimental groups, OVX, OVX+ICI (ICI), OVX+RLX (RLX) are presented. The effect of these experimental groups was examined in two different skeletal muscle types, the fast-twitch EDL and the slow-twitch SOL muscles, each selected because of their contrasting metabolic properties (i.e. fast-twitch muscles have greater glycolytic potential versus slow-twitch muscles which have lower glycolytic potential). *In vivo* muscle biopsies were taken (from the right leg) to determine what effect the experimental groups had on 'resting' muscle. An experiment was also performed to determine the metabolic response of these muscles to three hours of ischemia in all experimental groups (ischemia protocol performed on left leg). The metabolic data measured were glycogen, CP, ATP, and lactate as these variables represent metabolic pathways important to ischemic metabolism in skeletal muscle. Where statistically significant effects were observed, data were graphed to better illustrate the results.

3.3.a.(i). The role of skeletal muscle ER on *in vivo* CP levels in the fast-twitch EDL and slow-twitch SOL muscles.

In the fast-twitch EDL, *in vivo* CP levels of ICI females were 78% lower ($p \leq 0.001$) compared to OVX females and 77% lower ($p \leq 0.001$) compared to RLX (Figure 3.3.1). *In vivo* CP levels were not statistically different between OVX and RLX females.

As observed in the EDL, *in vivo* CP levels in the slow-twitch SOL illustrated similar results to ICI and RLX. *In vivo* CP levels of ICI females were 72% lower ($p \leq 0.001$) compared to OVX females and 69% lower ($p \leq 0.001$) compared to OVX females (Figure 3.3.1). *In vivo* CP
levels were not statistically different between OVX and RLX females.

3.3.a.(ii). The effect of fast- versus slow-twitch muscles on in vivo CP levels within each experimental group.

In vivo CP levels were significantly lower in the SOL compared to the EDL by 38% (p \leq 0.001) in OVX and 39% (p \leq 0.001) in RLX females (Figure 3.3.1, Table 3.3.1). In females who received ICI, in vivo CP levels were not statistically different between SOL and EDL muscles.
Figure 3.3.1. *In vivo* CP levels. Placebo (OVX), ICI 182,780 (ICI), Raloxifene (RLX). EDL, SOL. All values expressed as mean ± SD. \( a p \leq 0.05 \) OVX vs ICI within same muscle, \( b p \leq 0.05 \) ICI vs RLX within same muscle, \( * p \leq 0.05 \) vs EDL within same treatment group. n=6 in all groups.
3.3.b.(i). The role of skeletal muscle ER on *in vivo* glycogen levels in the fast-twitch EDL and slow-twitch SOL muscles.

In the fast-twitch EDL and slow-twitch SOL, *in vivo* glycogen levels were not statistically different among OVX, ICI, and RLX females (Table 3.3.1).

3.3.b.(ii). The effect of fast- versus slow-twitch muscles on *in vivo* glycogen levels within each experimental group.

*In vivo* glycogen levels were not statistically different between SOL and EDL muscles within OVX, ICI, and RLX females (Table 3.3.1).

3.3.c.(i). The role of skeletal muscle ER on *in vivo* ATP levels in the fast-twitch EDL and slow-twitch SOL muscles.

In both the fast-twitch EDL and slow-twitch SOL, *in vivo* ATP levels were not statistically different among OVX, ICI, and RLX females (Table 3.3.3).

3.3.c.(ii). The effect of fast- versus slow-twitch muscles on *in vivo* ATP levels within each experimental group.

*In vivo* ATP levels of the SOL were significantly lower compared to EDL muscles within each experimental group. Specifically compared to the EDL, *in vivo* ATP levels in the SOL were 36% lower (p≤0.001) in OVX, 34% lower (p≤0.001) in ICI, and 35% lower (p≤0.001) in RLX females (Table 3.3.3).
3.3.d.(i). The role of skeletal muscle ER on \textit{in vivo} lactate levels in the fast-twitch EDL and slow-twitch SOL muscles.

In both the fast-twitch EDL and slow-twitch SOL, \textit{in vivo} lactate levels were not statistically different among OVX, ICI, and RLX females (Table 3.3.4).

3.3.d.(ii). The effect of fast- versus slow-twitch muscles on \textit{in vivo} lactate levels within each experimental groups.

\textit{In vivo} lactate levels were not statistically different between SOL and EDL muscles in OVX, ICI, and RLX females (Table 3.3.4).
<table>
<thead>
<tr>
<th></th>
<th>Muscle</th>
<th>OVX</th>
<th>ICI</th>
<th>RLX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>EDL</td>
<td>96.3 ± 5.2</td>
<td>21.0 ± 5.3</td>
<td>89.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>60.2 ± 5.3 *</td>
<td>17.5 ± 2.4</td>
<td>55.5 ± 2.4 *</td>
</tr>
<tr>
<td><strong>3 Hours Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>EDL</td>
<td>6.6 ± 5.8</td>
<td>11.4 ± 2.3</td>
<td>10.2 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>5.2 ± 4.8</td>
<td>7.6 ± 2.8 *</td>
<td>8.6 ± 3.7</td>
</tr>
<tr>
<td><strong>Net Consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td>EDL</td>
<td>-89.7 ± 9.9</td>
<td>-6.1 ± 11.0</td>
<td>-79.4 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-55.0 ± 8.6 *</td>
<td>-9.9 ± 4.5</td>
<td>-46.9 ± 2.2 *</td>
</tr>
<tr>
<td><strong>% CP Consumed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EDL</td>
<td>-93.0 ± 6.4</td>
<td>-55.2 ± 7.1</td>
<td>-88.5 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-91.1 ± 8.7</td>
<td>-55.0 ± 19.6</td>
<td>-84.7 ± 6.2</td>
</tr>
</tbody>
</table>

**Table 3.3.1.** CP content (µmol/g dw) from *in vivo* and ischemic skeletal muscles. Placebo (OVX), ICI 182,780 (ICI), Raloxifene (RLX). Net CP consumption (calculated as *in vivo* CP minus 3 hour ischemic CP value), and % CP consumed (net CP consumption expressed as percentage of *in vivo* value) were calculated. Values are all expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs RLX within same muscle, * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
<table>
<thead>
<tr>
<th>GLYCOGEN</th>
<th>Muscle</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OVX</td>
</tr>
<tr>
<td><strong>In Vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol glucosyl units/g dw)</td>
<td>EDL</td>
<td>103.2 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>105.8 ± 14.5</td>
</tr>
<tr>
<td><strong>3 Hours Ischemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol glucosyl units /g dw)</td>
<td>EDL</td>
<td>6.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>18.9 ± 11.3  *</td>
</tr>
<tr>
<td><strong>Net Consumption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol glucosyl units /g dw)</td>
<td>EDL</td>
<td>-96.7 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-86.9 ± 9.5</td>
</tr>
<tr>
<td><strong>% Glycogen Consumed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EDL</td>
<td>-93.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>-82.9 ± 9.0  *</td>
</tr>
</tbody>
</table>

**Table 3.3.2.** Glycogen content (µmol glucosyl units/g dw) from *in vivo* and ischemic skeletal muscles. OVX female Sprague-Dawley rats who were given placebo (OVX), ICI 182,780 (ICI), and Raloxifene (RLX). Net glycogen consumption (calculated as *in vivo* glycogen minus 3 hour ischemic glycogen value), and % glycogen consumed (net glycogen consumption expressed as percentage of *in vivo* value) were calculated. Values are all expressed as mean ± SD. * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
Table 3.3.3. ATP content (µmol/g dw) of in vivo and ischemic skeletal muscles. Placebo (OVX), ICI 182,780 (ICI), Raloxifene (RLX). Net ATP consumption (calculated as in vivo ATP minus 3 hour ischemic ATP value), and % ATP consumed (net ATP consumption expressed as percentage of in vivo value) were calculated. Values are all expressed as mean ± SD. a p ≤ 0.05 vs OVX within same muscle, b p ≤ 0.05 vs RLX within same muscle, * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.

<table>
<thead>
<tr>
<th>ATP</th>
<th>Muscle</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O VX</td>
</tr>
<tr>
<td><strong>In Vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>31.4 ± 2.1</td>
<td>29.4 ± 0.9</td>
</tr>
<tr>
<td>SOL</td>
<td>19.8 ± 1.1 *</td>
<td>19.1 ± 1.4*</td>
</tr>
<tr>
<td><strong>3 Hours Ischemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>1.2 ± 0.6</td>
<td>1.3 ± 0.2 b</td>
</tr>
<tr>
<td>SOL</td>
<td>0.8 ± 0.06 *</td>
<td>1.3 ± 0.09 a,b</td>
</tr>
<tr>
<td><strong>Net Consumption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g dw)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>-30.2 ± 2.5</td>
<td>-28.1 ± 0.9 b</td>
</tr>
<tr>
<td>SOL</td>
<td>-19.2 ± 1.0 *</td>
<td>-17.8 ± 1.4 *</td>
</tr>
<tr>
<td><strong>% ATP Consumed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EDL</td>
<td>-96.1 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
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</tr>
<tr>
<td>LACTATE</td>
<td>Muscle</td>
<td>Experimental Group</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O VX</td>
</tr>
<tr>
<td>In Vivo (µmol/g dw)</td>
<td>EDL</td>
<td>5.7 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>3 Hours Ischemia (µmol/g dw)</td>
<td>EDL</td>
<td>235.4 ± 29.7</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>204.6 ± 21.8</td>
</tr>
<tr>
<td>Net Consumption (µmol/g dw)</td>
<td>EDL</td>
<td>+228.5 ± 27.5</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>+199.0 ± 22.2</td>
</tr>
<tr>
<td>% Lactate accumulated</td>
<td>EDL</td>
<td>+3622 ± 1039</td>
</tr>
<tr>
<td></td>
<td>SOL</td>
<td>+3783 ± 1188</td>
</tr>
</tbody>
</table>

Table 3.3.4. Lactate content (µmol/g dw) of in vivo and ischemic skeletal muscles. Placebo (OVX), ICI 182,780 (ICI), and Raloxifene (RLX). Net lactate accumulation (calculated as in vivo lactate minus 3 hour ischemic lactate value), and % lactate accumulated (net lactate accumulation expressed as percentage of in vivo value) were calculated. Values are all expressed as mean ± SD. * p ≤ 0.05 vs EDL within same experimental group. n=6 in all groups.
3.3.e.(i). The role of skeletal muscle ER on ischemic CP levels in fast- and slow-twitch muscles.

CP levels were significantly reduced after 3 hours of ischemia in all experimental groups, and the following describes the role of the ER on this response (Table 3.3.1).

In the fast-twitch EDL, ischemic CP levels were not statistically different among OVX, ICI, and RLX females (Table 3.3.1). The net EDL CP consumption during ischemia was significantly lower in ICI females by 93% (p≤0.001) compared to OVX females and 92% (p≤0.001) compared to RLX females (Figure 3.3.1), but the net EDL CP consumption during ischemia was not statistically different between OVX and RLX. The percent EDL CP consumption of was 41% lower (p≤0.001) in OVX and 38% lower (p≤0.001) in RLX compared to ICI females (Figure 3.3.1). The percent EDL CP consumption was not statistically different between OVX and RLX females.

Similar results to ICI and RLX on ischemic CP metabolism were also observed in the slow-twitch SOL muscle. Ischemic SOL CP levels were not statistically different among OVX, ICI, and RLX females (Table 3.3.1). The net SOL CP consumption during ischemia was 82% lower (p≤0.001) in OVX and 79% lower (p≤0.001) in RLX compared to ICI females (Figure 3.3.1), but the net SOL CP consumption during ischemia was not statistically different between OVX and RLX females. The percent SOL CP consumption was 40% lower (p≤0.001) in ICI compared to OVX. Although the percent SOL CP consumption was 35% lower in ICI females compared to RLX females, this was not statistically significant (Figure 3.3.1). The percent SOL CP consumption was not statistically different between OVX and RLX females.
3.3.e.(ii). The effect of fast- versus slow-twitch muscles on ischemic CP levels within each experimental group.

Although ischemic CP levels were not statistically different between SOL and EDL in OVX and RLX females, in females who were given ICI, ischemic CP levels were significantly lower in the SOL compared to the EDL by 32% (p=0.027) (Table 3.3.1). The net CP consumption during ischemia in the SOL was 39% lower (p≤0.001) in OVX and 41% lower (p≤0.001) in RLX females compared to EDL (Figure 3.3.1). The net CP consumption during ischemia was not statistically different between SOL and EDL of ICI females. The percent CP consumption was not statistically different between SOL and EDL in OVX, ICI, and RLX females (Figure 3.3.1).
Figure 3.3.2. (i) Net CP consumption (ii): % CP consumed during ischemia. Placebo (OVX), ICI 182,780 (ICI), Raloxifene (RLX). EDL, SOL. All values expressed as mean ± SD. \(^{a}\) p ≤ 0.05 vs OVX within same muscle, \(^{b}\) p ≤ 0.05 vs RLX within same muscle, * p ≤ 0.05 vs EDL within same treatment group. n=6 in all groups.
3.3.f.(i). The role of skeletal muscle ER on ischemic glycogen levels in fast- and slow-twitch muscles.

Glycogen levels were reduced after 3 hours of ischemia in all experimental groups, and the following describes the role of the ER on this response (Table 3.3.1).

In both the fast-twitch EDL and slow-twitch SOL, ischemic glycogen levels, the net glycogen consumption during ischemia, and the percent glycogen consumption during ischemia were not statistically different among OVX, ICI, and RLX females (Table 3.3.1).

3.3.f.(ii). The effect of fast- versus slow-twitch muscles on ischemic glycogen levels within each experimental group.

In the SOL, ischemic glycogen levels were 65% greater (p=0.026) in OVX and 70% greater (p=0.041) in ICI females compared to the EDL. RLX females had 72% greater ischemic glycogen values in the SOL compared to the EDL, but statistically, this was only a trend to significance (p=0.065). The net glycogen consumption during ischemia was not statistically different between SOL and EDL muscles in OVX, ICI, and RLX females. The percent glycogen consumption in the SOL was 12% lower (p=0.009) in OVX, 14% lower (p=0.010) in ICI, and 12% lower (p=0.015) in RLX females compared to the EDL.

3.3.g.(i). The role of skeletal muscle ER on ischemic ATP levels in fast- and slow-twitch muscles.

ATP levels were significantly reduced after 3 hours of ischemia in all experimental groups, and the following describes the role of the ER on this response (Table 3.3.3).

In the fast-twitch EDL, ischemic ATP levels were not statistically different between ICI and OVX and RLX and OVX females (Table 3.3.3), but ischemic ATP levels in ICI females
were 30% (p≤0.05) greater compared to RLX females. The net EDL ATP consumption during ischemia was not statistically different between OVX and ICI females; however, the net EDL ATP consumption was 7% (p≤0.05) lower in ICI females compared to RLX females, while no differences were observed between OVX and RLX females. The percent EDL ATP consumption of OVX and ICI females were not statistically different. There was a small (~1.4 %) yet significant difference (p≤0.05) in the percent EDL ATP consumption between ICI compared to RLX, while no differences were observed between OVX and RLX females.

In the slow-twitch SOL, ischemic ATP levels in ICI females were significantly greater by 38% when compared to both OVX (p≤0.001) and RLX (p≤0.001) females (Table 3.3.3). However, the net SOL ATP consumption during ischemia was not statistically different among OVX, ICI, and RLX females. There was a small (~3 %) yet significant difference in the percent SOL ATP consumption of ICI females compared to both OVX (p≤0.001) and RLX (p≤0.001) females (Table 3.3.3).

3.3.g.(ii). The effect of fast- versus slow-twitch muscles on ischemic ATP levels within each experimental group.

Although ischemic ATP levels were not statistically different between SOL and EDL in ICI and RLX females, ischemic ATP levels were 20% lower in the SOL compared to the EDL (p=0.041) in OVX females (Table 3.3.3). Compared to EDL, the net ATP consumption in the SOL was 37% lower (p≤0.001) in OVX, 36% lower (p=0.002) in ICI, and 37% lower (p≤0.001) in RLX females (Table 3.3.3). Compared to EDL, the percent ATP consumption in the SOL was 3% lower (p≤0.001) in ICI and 1% lower (p≤0.001) in RLX females, and no difference was observed in OVX females.
3.3.h.(i). The role of skeletal muscle ER on ischemic lactate levels in fast- and slow-twitch muscles.

Lactate levels were significantly increased after 3 hours of ischemia in all experimental groups, and the following describes the role of the ER on this response (Table 3.3.4).

In both the fast-twitch EDL and slow-twitch SOL, ischemic lactate levels, net lactate accumulation during ischemia, and the percent lactate accumulation during ischemia were not statistically different among OVX, ICI, and RLX females.

3.3.h.(ii). The effect of fast- versus slow-twitch muscles on ischemic lactate levels within each experimental group.

Lactate levels were increased after 3 hours of ischemia in all groups, but there were muscle specific responses. Ischemic lactate levels in the SOL were 22% lower (p≤0.001) in ICI and 19% lower (p=0.012) in RLX females, compared to the EDL. In OVX ischemic lactate levels were 13% lower in the SOL compared to the EDL, but this was a trend to significance (p=0.068) (Table 3.3.4). Compared to the EDL, the net SOL lactate accumulation during ischemia was 23% lower (p≤0.001) in ICI and 19% lower (p=0.013) in RLX females. In OVX females, the net lactate accumulation was lower in the SOL compared to the EDL, but this was a trend to significance (p=0.068) (Table 3.3.4). The percent lactate accumulation was not statistically different between SOL and EDL muscles in OVX, ICI, and RLX females.
Summary of Results for Study 3

*In vivo*:

ER blockade with ICI markedly decreased CP levels in either muscle. The selective ER agonist RLX had similar CP levels to OVX in either muscle. ER did not modulate glycogen, ATP or lactate levels in either muscle.

Ischemia:

ER blockade with ICI markedly decreased net CP consumption and the percent CP consumed in either muscle, which can be attributed to the markedly decreased *in vivo* CP levels in this group. ER blockade modestly decreased percent ATP consumption in both muscles, but the biological relevance of this is very minute because the difference between the group means was less than~ 3%. ER did not modulate ischemic glycogen and lactate profiles.
CHAPTER 4

DISCUSSION
4.1. STUDY 1: Regulation of metabolic enzymes by estradiol in fast-twitch skeletal muscles

Discussion of the data will focus on the relevant muscle enzymes that are modulated by estradiol. This will be followed by a brief discussion about why the other enzymes were not altered by estradiol. Finally, a synopsis of how the findings of this study contributed new information about the role played by estradiol in regulating the various energy producing pathways in fast-twitch skeletal muscle will be summarized with respect to what is known in the literature.

Estradiol’s control on the enzymes important to energy production were determined in 3 fast-twitch skeletal muscles. Each of the chosen muscles had differing proportions of fast-twitch fibers which have been documented in the literature (Appendix 1). Estradiol’s effects were most pronounced on the enzymes of glycogenolysis (GP) and glycolysis (PFK), with modest effects on CK and LDH, and lesser effects on mitochondrial enzymes that are affiliated with the Citric Acid Cycle (CS, SDH), fatty acid break down, and electron transport chain (COX). These effects occurred in fast-twitch muscles extensor digitorum longus (EDL) and semitendinosus, which have a greater proportion of type II fibers, as compared to the gracilis which has a lower proportion of fast-twitch fibers, supporting the hypotheses that estradiol's effects would be primarily observed in skeletal muscles with higher fast-twitch composition. The impact of estradiol on these particular enzymes is important because they are the key enzymes contributing to metabolic pathways for energy production (ATP) in fast-twitch muscles (Schiaffino & Reggiani, 2011). This is especially true when the metabolic demands for fast-twitch muscles are increased significantly, such as during high intensity exercise which requires rapid generation of energy from glycolytic resources (Schiaffino & Reggiani, 2011). The discussion will focus on the findings of the enzymes GP, PFK, LDH, and CK, which represent the enzymes in the order
of greatest to least significant effects of estradiol. This is followed by a review of why there were no significant effects observed on the oxidative enzymes CS, SDH, βHAD, and COX.

4.1.a. Glycogen Phosphorylase (GP)

In Study 1, the greatest effect of estradiol treatment in female rats was observed on GP, a key enzyme catalyzing the breakdown of glycogen in skeletal muscle. This work demonstrated for the first time that removing the majority of female sex hormones via OVX increased GP activity in muscles with the highest proportion of fast-twitch fibers. When OVX females were given estradiol, GP activity returned to levels observed in intact females, confirming estradiol is responsible for decreasing the activity of the key enzyme responsible for glycogenolysis. This finding compliments the reports on estradiol's effect on glycogen metabolism in skeletal muscle, wherein estradiol treatment resulted in a reduction of glycogen use in rat skeletal muscle during sub-maximal exercise, termed the 'glycogen-sparing effect' (Kendrick et al., 1987). The fast-twitch red vastus lateralis muscles of these females given estradiol had glycogen levels that were decreased by only 22% compared to 70% in control females at the end of the exercise duration, which was similarly observed in the fast-twitch white vastus lateralis muscle (Kendrick et al., 1987), indicating that these fast-twitch muscles responded to estradiol's glycogen-sparing effect. Even male rats given 17β-estradiol for 5 days and exercised up to 2 hours demonstrated glycogen-sparing in both red and white vastus lateralis muscles (Rooney et al., 1993). Human studies also suggest that estradiol can depress glycogen use. Women in the luteal phase of the ovarian cycle (associated with high estradiol and progesterone levels) and who performed submaximal exercise for an hour demonstrated that their vastus lateralis used 25% less glycogen compared to women in the follicular phase of the ovarian cycle (associated with mid-ranged estradiol, very low progesterone levels; Hackney, 1999). Based on the above observations, the
mechanism by which estradiol suppresses glycogen-use during sub-maximal exercise in fast-twitch muscles can be attributed to estradiol reducing GP activity as illustrated by this thesis work.

It was also hypothesized that the effect of estradiol on GP activity would occur to a greater extent in muscles that had a greater fast-twitch fiber composition. Work in this study supports this hypothesis, since the effect of estradiol on GP activity was statistically significant in the SMT and EDL muscles which have approximately 94% and 96% total type II fast-twitch fibers (Delp & Duan, 1996), versus the GRAC which elicited a trend to significance and has approximately 80% total type II fast-twitch fibers (Delp & Duan, 1996). Choosing the lowest hormone group, OVX, skeletal muscle comparisons showed that GP activity was different among the 3 fast-twitch muscles. Specifically, GP activity was 30% greater in the EDL which has the highest fast-twitch fiber proportion compared to the GRAC which has the lowest (Table 3.1.1). This may suggest that even within fast-twitch muscles, increases in GP activity are associated with higher fast-twitch fiber content. In the presence of estradiol, either cycling estradiol (INT) or continuous estradiol treatment (ET), fiber type differences in GP activity were not observed (Figure 3.1.2). Thus, these differences in GP activity among the fast-twitch muscles suggest that fast-twitch muscles with greater total type II fibers illustrated significant effects of estradiol compared to fast-twitch muscles with fewer total type II fibers.

Recent work has suggested that GP activity may be controlled by estradiol at the nuclear level. The various mRNA levels of metabolic enzymes in the vastus lateralis of women who were in the mid-follicular and the mid-luteal phase of the menstrual cycle were studied (Fu et al., 2009). Their study showed that GP mRNA content in the follicular phase was approximately 1.6 fold greater compared to the luteal phase. This suggests that estradiol may have an inhibitory role in regulating mRNA expression of GP and this could translate to lower GP protein levels and
may explain how GP activity in this thesis study was suppressed.

4.1.b. Phosphofructokinase (PFK)

In Study 1, the most remarkable effect of estradiol on PFK activity was observed in the SMT muscle, with no significant effects in the GRAC and EDL muscles. There was a trend for PFK activity to be increased when females were ovariectomized in the SMT muscle, and treatment with estradiol resulted in a significant reduction in PFK activity compared to OVX females. These data suggests that estradiol reduced PFK activity in the SMT muscle as per Hypotheses 1 which held that estradiol would decrease the activity of enzymes involved in glycolysis. However, it was also hypothesized that the effect of estradiol would be greater in muscles with greater fast-twitch composition. Though no effect was observed in GRAC (lowest proportion of fast-twitch), estradiol did reduce PFK activity in the SMT (higher proportion of fast-twitch fibers), but not in the EDL. Since the two latter muscles have approximately 94% and 96% total fast-twitch fibers, respectively (refer to Appendix 1), this results suggests that the effect of estradiol on PFK activity in the EDL was blunted. Interestingly, Beckett et al. (2002) showed that female rats given 3 weeks of estradiol did not show altered PFK activity in the vastus medialis (fast-twitch muscle), which along with the findings of Study 1, illustrate that estradiol does not play a strong or consistent role on this enzyme's activity in fast-twitch muscles. What mechanism estradiol utilizes to selectively regulate PFK in different fast-twitch muscles is currently unknown.

An interesting pattern among the fast-twitch muscles occurred in the OVX and ET groups. When examining the lowest hormone group, OVX, skeletal muscle comparisons showed that PFK activity was different among the 3 fast-twitch muscles. Specifically, PFK activity was 36% greater in the SMT and 30% greater in the EDL which have the highest fast-twitch fiber
proportion compared to the GRAC which has the lowest (Table 3.1.1). A similar pattern was also observed in GP activity, and as discussed earlier, this may suggest that even within fast-twitch muscles, increases in PFK activity are associated with higher fast-twitch fiber content. In the presence of estradiol, either cycling (INT) or continuous estradiol (ET), fiber type differences in PFK activity were not observed (Figure 3.1.1). Thus, these differences in PFK activity among the fast-twitch muscles suggest that fast-twitch muscles with greater total type II fibers will have greater effects of estradiol compared to fast-twitch muscles with fewer total type II fibers.

4.1.c. Role Transcription Factor Nur77 may have in regulating GP and PFK activity

One reason estradiol may have decreased GP activity (SMT, EDL) and PFK activity (EDL) in these fast-twitch muscles may be related to transcription factors that regulate glucose metabolism. The NR4A orphan nuclear receptor Nur77, a transcription factor from the nuclear receptor super family, activates the expression of the genes that regulate glucose metabolism in skeletal muscle, such as GP and PFK (Chao et al., 2007). The promoter site for GP and PFK have a consensus Nur77 binding site that Nur77 utilizes to increase GP and PFK expression, and Nur77 is expressed significantly greater in fast-twitch muscles (i.e. EDL, tibialis anterior) compared to the slow-twitch muscles (SOL) (Chao et al., 2007). Estradiol, acting via ERα, has been shown to physically interact with Nur77 in Leydig cells of the testes, and that the ERα has an inhibitory effect on steroidogenesis by inhibiting the DNA-binding activity of Nur77 (Lee et al., 2012). This resulted in a suppression of Nur77's ability to stimulate the transcription of steroidogenic enzyme genes. The study by Lee et al. (2012) also showed that estradiol activation of ERα down-regulated Nur77 protein levels. If the interaction between estradiol and Nur77 in Leydig cells also occurs in fast-twitch muscles, the mechanism illustrated in Figure 4.1 could explain how estradiol effected GP and PFK activity in muscles with greater Type II fiber
composition in this thesis. Since Nur77 is expressed to a greater extent in fast-twitch muscles versus slow-twitch muscles (Chao et al., 2007), and that Nur77 can increase the expression of genes for GP and PFK, then theoretically, the reduction in GP and PFK activity observed with estradiol treatment in Study 1 could be mediated by effects through Nur77. Theoretically, estradiol may inhibit Nur77 protein levels in fast-twitch muscles with the greatest proportion of fast-twitch fibers, and that this may be via the ERα isoform which has been shown to exist in skeletal muscle (refer to Introduction 1.3.b). The combined effects of estradiol via ERα inhibiting Nur77 function in fast-twitch muscles could lead to reduced GP and PFK mRNA levels, which would result in reduced translation of these mRNA to protein. This would result in lower protein levels of these enzymes, thereby reducing the activity of GP and PFK. Future work could determine if the significant effects of estradiol on GP and PFK activities in these fast-twitch muscles are attributed to Nur77.
Figure 4.1. Proposed potential mechanism illustrating how estradiol (E2) binding to ERα could reduce GP and PFK activity levels in fast-twitch muscles via down-regulation of Nur77 expression.

4.1.d. Lactate Dehydrogenase (LDH)

Estradiol did not appear to alter LDH activity in any of the fast-twitch muscles studied, suggesting that LDH is not regulated by estradiol in these muscles. However, unlike the previous enzymes discussed above, OVX resulted in a reduction in LDH activity that was not attributed to estradiol in the GRAC muscle. Thus, the reduction in LDH activity in the GRAC could be due to other ovarian steroid hormones that are removed with OVX, such as progesterone. It should be noted in the SMT and EDL, a sample size of 10 would make the LDH data significant since a trend to significance for estradiol to reduce LDH activity in the SMT (p=0.058) and EDL
125

(p=0.054) muscles was observed. Although skeletal muscle effects of estradiol on LDH activity are unknown, other published data report that estradiol can increase the transcription of one LDH isoform in breast cancer cells (Li et al., 2004) and increase LDH activity in normal mammary tissue (Richards & Hilf, 1972). Thus, the effects of estradiol on LDH activity are organ specific and need to be considered when evaluating its role on LDH activity.

LDH is the enzyme that catalyzes the reversible reaction of pyruvate to lactate and is generally greater in fast-twitch muscles compared to slow-twitch muscles (refer to Table 1.2). The work in Study 1 demonstrated fiber type differences in the lowest hormone group (OVX). As previously demonstrated in the PFK and GP data of OVX females, LDH activity was 26% greater in the SMT which has the greater fast-fiber proportion compared to the GRAC which has the lowest (Table 3.1.1). Estradiol eliminated the fiber type effect, which was similarly observed in the GP and PFK data. This observation has not been previously reported for skeletal muscle. It could be speculated that in the OVX group, since there are no sex hormone effects, muscle type differences in enzyme activity are more evident. In OVX females, reasons for why the GRAC would consistently have lower activity of GP, PFK, and LDH compared to the SMT may be attributed to their lower proportion of total Type II fibers (Appendix 1). Recall that Type II fibers generally have greater glycolytic capacity compared to Type I fibers, and muscles with lower Type II fibers would predispose them to a lower glycolytic potential.

4.1.e. Creatine Kinase (CK)

CK is the only enzyme that catalyzes the reaction that transfers the phosphoryl group on CP to ADP, rapidly producing ATP and creatine. This process is especially important in fast-twitch muscles where their demand for ATP can be increased rapidly. To date, the role estradiol has on CK activity in skeletal muscle has not been reported. In Study 1, the muscle with the
lowest total Type II fibers, GRAC, demonstrated that CK activity differed between INT and OVX females. Specifically, CK activity was 33% lower in OVX compared to INT females. CK activity was similar between INT and ET, suggesting that estradiol reduced CK activity to INT female levels. On the other hand, CK activity was also similar between OVX and ET, and a sample size calculation determined that a sample of 11 would make the difference between OVX and ET females significant. Intriguingly, the effect of estradiol on CK activity was not observed in any of the fast-twitch muscles studied, suggesting that CK is not regulated by estradiol in fast-twitch muscles with greater total Type II fibers and these results are the first to be reported in the literature. Similar to the observations for LDH activity, OVX resulted in a reduction in CK activity that was not attributed to estradiol in the GRAC muscle. The reduction in CK activity in the GRAC could be due to other female sex hormones that are removed with OVX such as progesterone.

4.1.f. Citrate Synthase (CS), Succinate Dehydrogenase (SDH), βHAD (beta-hydroxyacyl CoA dehydrogenase), and Cytochrome C Oxidase (COX)

The results of Study 1 report that CS activity was unaffected by estradiol in all 3 fast-twitch muscles, which supports the hypotheses. CS was studied because it is the key regulatory enzyme for the Citric Acid Cycle and had the potential to be a site for regulation by estradiol. In Study 1, CS levels were low in all three fast-twitch muscles. As reviewed in Table 1.2 and in Appendix 1, there is a range of CS activity reported by other papers in different types of fast-muscles and slow-twitch muscles, which may be attributed to the species (human versus rat) and assaying techniques as the units of CS activity are expressed differently. In Table 1.2, CS activity is usually greater in slow-twitch muscles or in Type I fibers compared to lower activities in fast-twitch or Type II fibers. Although it is difficult to directly compare the values from Study 1 to
those in the literature, the CS values of Study 1 fit into the trend of lower CS values observed in fast-twitch muscles that have a lower capacity for oxidative metabolism. The effect of estradiol on CS activity was investigated by Campbell & Febbraio (2001) who demonstrated that estradiol did not alter CS activity in the red and white *gastrocnemius* muscle (fast-twitch) of female rats despite CS activity being approximately 60% greater in the red compared to the white *gastrocnemius*. In another fast-twitch muscle, *vastus medialis*, CS was increased significantly by 16% in estradiol treated female rats (Beckett et al., 2002). Thus, estradiol is selective in how it regulates CS activity in fast-twitch muscles. It could be speculated that fiber type distribution does not determine the effect of estradiol on CS activity since the fiber type distribution of the GRAC, SMT, EDL, and the muscles mentioned above have varying degrees of Type I and the three Type II fibers (see Appendix Table 1 and 2).

Another key regulator of the Citric Acid Cycle is SDH which demonstrated that estradiol did not alter its activity in any of the 3 fast-twitch muscles studied, which supports the hypotheses, and is the first time to be reported. SDH was studied because it is embedded in the inner mitochondrial membrane and forms a direct association between the Citric Acid Cycle and the electron transport chain (Berg et al., 2002). Only recent research has identified the genetic factors that encode the gene for SDH (Rutter et al., 2010); however, what role estradiol or other hormones, such as progesterone, have in this process in skeletal muscle has yet to be elucidated.

Estradiol did not alter βHAD activity among the three fast-twitch muscles of Study 1, which suggests that βHAD is not regulated by estradiol in these muscles, supporting the hypotheses. Work from Study 1 together with that from the literature indicate that muscle fiber distribution may be critical in understanding how estradiol controls βHAD in skeletal muscle. βHAD catalyzes the third reaction of β-oxidation of fatty acids by converting L-3-hydroxyacyl CoA and NAD⁺ to 3-ketoacyl CoA + NADH + H⁺ (Berg et al., 2002). Generally, βHAD has
greater activity in slow-twitch skeletal muscles or in fast-twitch muscles that are 'red' (see Table 1.2). In fast-twitch muscles, βHAD activity regulation by estradiol may be dependent on fast-twitch fiber distribution of Type IIa, IID/X, and IIb, which supports the theory that estradiol controls βHAD in those fast-twitch muscles that have a greater dependence for fatty acid oxidation as a source of energy. In female rats, βHAD activity was not affected by either OVX or estradiol in white *gastrocnemius* (Campbell & Febbraio, 2001) and *vastus medialis* muscles (Beckett *et al.*, 2002). It is important to note that the white *gastrocnemius* and *vastus medialis* are fast-twitch muscles that have a high proportion of Type IIb fibers, ~92% and ~62%, respectively (see Appendix 1). On the other hand, βHAD activity was reduced in OVX rats which was restored to gonadally intact female values with estradiol in the red *gastrocemius* muscle (Campbell & Febbraio, 2001). Estradiol may affect βHAD activity to a greater degree in the red *gastrocemius* muscle because it has a greater distribution of Type I and IIa fibers that rely primarily on oxidative metabolism (see Appendix 1). The muscle differences in the potential for β-oxidation was also evident, in that the red *gastrocemius* had approximately 76-79% greater βHAD activity compared to the white *gastrocemius* in each of the treatment groups (Campbell & Febbraio, 2001), confirming the observation that fast-twitch muscles with greater βHAD activity will be more responsive to estradiol. In Study 1, βHAD activity was relatively low (ranging from 1.5 -3.5 mol/kg protein/hr), indicating a low potential for fatty acid breakdown among these muscles, which may explain why estradiol did not affect βHAD activity in the fast-twitch muscles of Study 1.

COX is the last enzyme complex of the electron transport chain that catalyzes the conversion of oxygen and reduced cytochrome c to water and oxidized cytochrome c (Quinzii & Hirano, 2010). Estradiol did not alter COX activity in any of the fast-twitch muscles studied, suggesting that COX is not regulated by estradiol in these fast-twitch muscles, supporting the
hypotheses and is the first to be reported in the literature.

**Figure 4.2.** Summary of significant findings of Study 1 which examined the effect of estradiol on key enzymes involved in energy production in the fast-twitch muscles *gracilis*, *semitendinosus*, and *extensor digitorum longus* muscles. The enzymes GP and PFK and their respective arrows are coloured in red to indicate a significant estradiol effect. All other enzymes are written in grey to indicate no effect of estradiol. Font sizes for the enzymes were selected to visually approximate the relative amount of activity of enzyme in fast-twitch muscles.
Summary of Discussion for Study 1

Study 1 demonstrated that in fast-twitch muscles, estradiol reduced GP activity and this occurred significantly in fast-twitch muscles with the highest proportion of total Type II fibers (SMT, EDL). Estradiol significantly reduced PFK activity in the SMT muscle, but not in the EDL, which also has a high total Type II content, suggesting that other regulating mechanisms may determine how estradiol controls metabolism in these fast-twitch muscles. In the GRAC, CK and LDH activity were reduced by OVX, which was not due to decreased estradiol, suggesting other hormones, such as progesterone, may regulate CK and LDH activity. The key regulatory enzymes of the Citric Acid Cycle (CS and SDH), fatty acid oxidation (βHAD), and the electron transport chain (COX) were not regulated by estradiol in all three fast-twitch muscles. Since muscle-fiber type differences were observed in response to estradiol, and because estradiol significantly influenced both the breakdown of glycogen and glycolysis, this work has established the platform for the next series of experiments in Study 2, focusing on metabolism and responses to stress, such as a lack of blood flow (ischemia).
4.2. STUDY 2: The role of intermittent-estradiol and progesterone in skeletal muscle -
effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

Based on the findings of Study 1 which illustrated that there were muscle type differences in how estradiol influenced GP and PFK activity, the work of Study 2 further investigated this characteristic by examining the differences between the muscle with the greatest proportion of fast-twitch fibers (EDL) and slow-twitch fibers (SOL). Additionally, to test the metabolic responses to estradiol which were demonstrated in Study 1, an ischemic model was utilized to force the tissue to be more reliant on anaerobic sources of energy production. As reviewed in the Introduction, glycogenolysis (breakdown of glycogen) will provide the glycosyl units which are the substrates glycolysis requires for energy production (ATP), resulting in the end product lactate. Thus, glycogen, ATP, and lactate levels were assessed. CP was also measured since it is an important source of ATP in skeletal muscles and Study 1 indicated that OVX reduced CK activity; however, although estradiol was shown not to be responsible for this reduction, it was speculated that progesterone may play a role. Thus, Study 2 investigated the effects of both estradiol and progesterone in two different types of skeletal muscles (EDL and SOL), and the responses of these muscles to estradiol and progesterone under ischemic duress.

4.2.a. In vivo effects of intermittent estradiol and progesterone

The hypotheses predicted that intermittent estradiol would increase and that progesterone would decrease in vivo glycogen levels, and that this would occur to a greater extent in the fast-twitch muscle with the greatest proportion of fast-twitch fibers (EDL) from Study 1 as compared to the predominately slow-twitch muscle (SOL). The Study 2 data affirmed parts of these hypotheses, but intermittent estradiol did not increase in vivo glycogen levels in either muscle.
Reasons for this may be attributed to the model of intermittent estradiol. As discussed in Section 2.2.b, a model of cycling estradiol was examined in female rats which was achieved by giving chronic exposure to estradiol for 2 weeks, followed by 6 weeks of low estradiol, over a 120 day time span. In other studies that reported estradiol increasing \textit{in vivo} glycogen levels in skeletal muscle, longer treatment durations were utilized, such as 10 weeks (Puah & Bailey, 1985) and 15 weeks (Ahmed-Sorour & Bailey, 1981) of chronic estradiol treatment. Thus, it could be speculated that this intermittent estradiol model did not maximize the estradiol response required to increase \textit{in vivo} glycogen levels. Progesterone alone reduced \textit{in vivo} glycogen levels significantly by 25\% compared to OVX females and this occurred significantly only in the slow-twitch SOL, which is opposite to what was hypothesized. \textit{In vivo} glycogen levels were only reduced in the EDL when intermittent estradiol treatment was given concomitantly with progesterone. This might suggest that there are fiber type differences in response to progesterone in the fast-twitch muscle type that are enhanced by intermittent estradiol. To date, these findings are the first evidence to report muscle fiber type differences in response to intermittent estradiol and progesterone on skeletal muscle glycogen levels and identify differences in their response to progesterone administration.

The mechanism by which progesterone mediates its effects in skeletal muscle have yet to be elucidated in the literature. Work from pregnancy studies suggest that gestational insulin-resistance at the level of skeletal muscle may be due to progesterone (Gras \textit{et al.}, 2007; Leturque \textit{et al.}, 1989; Kalkoff, 1982). Additionally, the metabolic effects of progesterone in skeletal muscle have been described to be the opposite of those exerted by estradiol and that insulin-mediated glucose uptake is reduced by progesterone (Puah & Bailey, 1985; Kalkoff, 1982). Puah & Bailey (1985) showed that when estradiol was administered to OVX mice, the rate of insulin-stimulated glucose uptake and glycogen formation was significantly increased in the slow-twitch
SOL while progesterone administration did not alter these parameters. They also showed that when both hormones were administered, the estradiol effect was removed, which the authors described as “progesterone antagonized the estrogen effect” (Puah & Bailey, 1985). Additionally, fast-twitch muscles and comparisons to the SOL were not executed to investigate muscle fiber type differences in these publications. Thus, the effect of progesterone reported in Study 2 agrees with what has been reported in the literature with regard to information presented for the SOL, and additionally this establishes what should happen under ischemic stress.

However, another important contribution of the information from Study 2 is that the SOL data were different compared to another fiber type of contrasting metabolic properties (EDL), which has not been reported in publications concerning the effects of progesterone. This is important because muscle fiber differences were observed in Study 2, as discussed below.

One of the interesting findings of Study 2 is the way progesterone reduced \textit{in vivo} glycogen levels in the two different types of skeletal muscle. In Study 2, progesterone alone did not alter \textit{in vivo} glycogen levels in the EDL, compared to that of the SOL which had approximately 25% lower \textit{in vivo} glycogen levels. However, when intermittent estradiol and progesterone were given, \textit{in vivo} glycogen levels in the EDL were reduced by approximately 25%. This is in contrast to the SOL because progesterone alone reduced \textit{in vivo} glycogen levels and this effect was maintained with intermittent estradiol. What caused these muscle specific responses to progesterone are not clear. Gras \textit{et al.} (2007) reported that glycogen content of SOL was decreased when incubated in progesterone, which was independent of insulin. Additionally, Gras \textit{et al.} (2007) reported that similar results were observed with the EDL, but these data were not published in their paper nor were they statistically compared to the SOL. It is possible that progesterone alters the balance between glycogen synthesis and glycogen consumption, which would regulate the level at which glycogen is sustained, and this may have differed between the
two types of muscles. Leturque et al. (1989) demonstrated that progesterone reduced the rate of glycogen synthesis in both the slow-twitch SOL and fast-twitch EDL. Similarly, SOL muscles incubated in progesterone had reduced rates of glycogen synthesis (Gras et al., 2007), but this was not studied in the EDL to further clarify muscle fiber type differences in response to progesterone. Thus, these papers indicate that progesterone shifts the balance in the regulation of glycogen levels in skeletal muscle, decreasing glycogen synthesis, which may explain why glycogen levels in Study 2 were decreased by progesterone in the SOL. However, this information does not explain why in Study 2 progesterone did not decrease in vivo glycogen levels in the EDL. Additionally, it is currently unknown what role progesterone may have on the rate of glycogen degradation, since the balance between glycogen synthesis and degradation both contribute to glycogen levels.

Compared to estradiol, information regarding progesterone's role and mechanism in glucose metabolism is limited, especially in different types of skeletal muscle. Although there is controversy on the existence of PR in skeletal muscle, a progesterone receptor membrane component 1 (PGRMC-1) has been shown to exist in skeletal muscle. PGRMC-1 is theorized to be involved in mediating some effects of progesterone in progesterone responsive tissues; however, it is important to note that progesterone does not directly bind to PGRMC-1 (Rohe et al., 2009; Cahill et al., 2007). PGRMC-1 is expressed in the ovaries (granulosa and luteal cells) (Peluso et al., 2012) where its key functions are to protect cells from DNA damage by binding to heme found in P450 proteins (as heme is involved in damage resistance), and to participate in cholesterol synthesis (Rohe et al., 2009). Interestingly, Gras et al. (2007) demonstrated that the mRNA levels of PGRMC-1 were significantly greater in the SOL compared to the EDL. Although the downstream effects of PGRMC-1 are not fully understood, it is speculated that in skeletal muscle, PGRMC-1 may be involved in how progesterone mediates its effects on...
glycogen metabolism especially since fiber type differences exist in its expression.

The data in Study 2 are to the author's knowledge the first evidence of an effect of intermittent estradiol and progesterone on skeletal muscle CP levels in contrasting types of muscle types, fast- and slow-twitch muscles. Intermittent estradiol did not significantly alter in vivo CP levels in either muscle type, which was also observed when progesterone alone was administered to OVX females. When both hormones were given, significant reductions in in vivo CP levels were observed in the EDL and SOL compared to controls. Unlike the in vivo glycogen data, progesterone alone did not significantly reduce CP levels in the SOL, although this value was 20% decreased in progesterone treated females compared to OVX females (trend to significance). Interestingly, CP levels were significantly decreased by 17% in the EDL, but only when compared to intermittent estradiol females. Only when both intermittent estradiol and progesterone were given together were reductions in CP levels observed in both the EDL and SOL. The lower CP levels in response to progesterone in both the EDL and SOL muscles suggest that the need for energy via CP breakdown was either enhanced or less CP was synthesized, as the balance to sustain CP is also dependent on its synthesis and breakdown in these muscles. Gras et al. (2007) also reported that progesterone may "shift from the aerobic towards the anaerobic pathway" because progesterone reduced pyruvate and palmitate (fatty acid) oxidation, thereby inhibiting energy producing pathways via oxidative phosphorylation.

Thus their findings, along with those of Study 2, illustrate that progesterone may change skeletal muscle metabolism to rely more on non-oxidative sources of energy production via increased CP consumption, occurring in both fast- and slow-twitch muscles. The implications of progesterone reducing in vivo CP levels in skeletal muscle would suggest that these muscles have fewer sources of energy available to them from CP. This could potentially render these muscles at a metabolic disadvantage during times of stress when sources of ATP from CP are a high
priority, such as during ischemia.

The sex hormones estradiol and progesterone did not result in alterations in the *in vivo* ATP levels of either muscle type. Overall, intermittent estradiol did not alter *in vivo* ATP levels in either the EDL or the SOL. Progesterone did not change *in vivo* ATP levels in either muscle when compared to OVX females. Although *in vivo* ATP levels were significantly lower by 23% in the SOL of females given progesterone relative to intermittent estradiol females, this difference did not exist when both hormones were given. This indicates that despite the significantly lower *in vivo* glycogen and CP levels due to intermittent estradiol and progesterone in both muscle types, energy levels were not significantly altered by these hormones. It can be implied that other sources of ATP, via oxidative phosphorylation, were able to sustain the energetic requirements of the muscles. This is especially true since lactate levels were low and within the physiologic range, and also were not altered either by intermittent estradiol and progesterone.

### 4.2.b. Effect of intermittent estradiol and progesterone on ischemic muscle metabolism

Investigating the effect of intermittent estradiol and progesterone on skeletal muscle responses to ischemia and the effectiveness of these hormones in different types of skeletal muscle is a novel area of study. Ischemia was chosen as a metabolic stress model to test the adaptations of skeletal muscles to these hormones. Prior to this study, there were no published studies examining the role of estradiol and progesterone under ischemic conditions in various kinds of skeletal muscle. In the model of global ischemia used in Study 2, the lack of tissue perfusion impeded oxygen & nutrient delivery and also prevented wash-out of metabolic end products. This ischemic stress forced the skeletal muscle to utilize the two metabolic pathways available for ATP production under these conditions: (1) CP break down and (2) anaerobic
glycolysis, which relies primarily on substrate (glycosyl units) stored as glycogen in the cell (Walker, 1991). Because the hormonal adaptations observed in Study 1 demonstrated that estradiol significantly altered glycogen phosphorylase (GP) activity and PFK activity, the key enzymes important in ischemic skeletal muscle metabolism, studying ischemia was a logical model to test these metabolic adaptations (Figure 4.1).

The hypotheses predicted that intermittent estradiol and progesterone will decrease glycogen consumption to a greater degree in fast-twitch muscles, but the hypothesized mechanisms with which these two female sex hormones would accomplish this differed. Glycogen breakdown during ischemia can be impaired either by decreasing the activity of glycogenolytic enzymes, or by reduced availability of glycosyl units stored in glycogen. It was hypothesized that intermittent estradiol would cause glycogen-sparing during ischemia due to decreased activity of GP, the enzyme of glycogen breakdown, as per Study 1. In contrast, progesterone was hypothesized to cause glycogen-sparing during ischemia due to reduced availability of substrate via decreased \textit{in vivo} glycogen levels. Although the hypothesis stated the hormonal effects would occur greatest in the EDL, the data do not support this. The EDL showed no response to hormones at 3 hours of ischemia. In the SOL, ischemic glycogen levels with intermittent estradiol were not statistically different from OVX females, but ischemic glycogen levels in intermittent estradiol females were 30% greater compared to OVX. Intriguingly, the SOL demonstrated that the ischemic glycogen levels at 3 hours of ischemia were significantly greater in the intermittent estradiol group compared to progesterone, but when both intermittent estradiol and progesterone combined were given, no effects were observed. Based on these data, it could be concluded that neither intermittent estradiol nor progesterone significantly altered the ischemic glycogen responses in both muscle fiber types. However, the ischemic glycogen values being compared are only the absolute values of glycogen at the end of the 3 hour ischemic period.
and *in vivo* glycogen levels were different among the treatment groups. This is important because the level of glycogen present in muscle is a factor in determining the degree of glycogenolysis. To correct for this, the net amount of glycogen consumed was calculated to adjust for any differences *in vivo* glycogen levels may have had in determining ischemic responses.

To further evaluate if the differences in ischemic glycogen levels resulted in glycogen-sparing in both the EDL and SOL, the net glycogen consumption during ischemia was calculated (Figure 3.2.5). If there was a glycogen-sparing effect, this would result in lower net glycogen consumption value compared to other groups. As found in Study 2, intermittent-estradiol did not induce glycogen-sparing in either muscle. However progesterone administration in the SOL induced glycogen-sparing, with approximately 25% less net glycogen consumption compared to OVX. Interesting, this effect in the SOL was sustained even when intermittent estradiol was given with progesterone. The EDL was unresponsive to the effects of progesterone and only when administered with intermittent estradiol did glycogen-sparing occur, achieving approximately 25% lower net glycogen consumption compared to muscles of OVX females. Thus, these findings strongly support the need to calculate the net glycogen consumption during ischemia in order to properly evaluate the glycogen-sparing effect in skeletal muscle. Relying solely on the absolute ischemic glycogen levels would have led to incorrect conclusions of intermittent estradiol and progesterone on this response. Another important impact of these data is that this is the first and only report of progesterone's glycogen-sparing effect during ischemia in skeletal muscle. Although Gras *et al.* (2007) did not study ischemia, they did elucidate that glucose oxidation, glucose transport, glycogen synthesis, and glycogen content were decreased by progesterone in the SOL. If progesterone reduces the availability of glucose entry, this would result in reduced storage of glucose (as glycogen), explaining the lower glycogen levels found by both Gras *et al.* (2007) and here in Study 2. Thus, the Study 2 data support the hypothesis that
progesterone exerts glycogen-sparing, but this was independent of muscle fiber type. Another possible level of control progesterone may have had is on the ability of the muscles to consume glycogen. Specifically, not only can the net glycogen consumption be reduced, but also the flux to breakdown glycogen. Therefore, in order to address that variable, the net glycogen consumption was adjusted as a percentage of the \textit{in vivo} values.

The relevance of the adjusted net consumption of glycogen was illustrated in Figure 3.2.5. When looking at the EDL, there was no significant difference in percent glycogen consumed during ischemia, and all experimental groups consumed \(>95\%\) of the glycogen available. This is an intriguing finding because in the EDL of females who received IE+P, the net glycogen consumption was significantly lower by 23\% compared to females who received IE or OVX (Figure 3.2.5). This indicates that despite the lower net consumption of glycogen due to progesterone, the ability to consume this glycogen was not impaired since more than 95\% of it was utilized, similar to that of OVX and IE females. This is an important finding because this information supports the hypotheses that progesterone would have a glycogen-sparing effect due to an inability to store glycogen and not in the ability to consume glycogen. In the SOL, a unique ischemic glycogen consumption pattern was uncovered, especially in intermittent estradiol females. In the SOL and only when compared to OVX, neither intermittent estradiol, progesterone, nor the combination of both hormones changed the adjusted net glycogen consumption. Interestingly, intermittent estradiol had the lowest mean adjusted net glycogen consumption of all the experimental groups; however, this was only statistically different compared to progesterone treated females whereby intermittent estradiol females consumed only 73\% of glycogen during ischemia, compared to progesterone females who consumed 87\% (Figure 3.2.5.). Hence, these data suggest that intermittent estradiol causes less glycogen consumption from the available glycogen \textit{in vivo} during ischemia compared to progesterone. It is
difficult to conclude that intermittent estradiol resulted in a glycogen-sparing effect during ischemia as this observation was not statistically different compared to controls. It could be speculated that the reason intermittent estradiol treated females demonstrated this effect was due to lower GP activity (as shown in Study 1), thereby impairing glycogenolysis and the availability of substrate for glycolysis.

Overall, CP levels were completely utilized within the ischemic duration in both types of muscles, and as expected, all muscles experienced 80-90% CP consumption, with the EDL having a greater net CP consumption compared to the SOL, irrespective of the treatment group. In both muscles, intermittent estradiol and progesterone did not alter ischemic net CP consumption compared to OVX females. However in the EDL, when both hormones were given together, the net CP consumption was significantly lower by 20% compared to controls, which could be attributed to the lower in vivo CP levels of this group, which had 19% lower levels compared to controls. In the slow-twitch SOL, a similar response was observed, but it was a trend to significance. It may be that the EDL's greater CP reserve relative to the SOL exacerbated any effect that estradiol and progesterone had on CP consumption during ischemia. This result strongly suggests that starting with a lower in vivo reserve of energy as CP impacts CP consumption. Nevertheless, adjusting net CP consumption during ischemia illustrated that both muscles were not affected by intermittent estradiol, progesterone, or both administered together, indicating that the ability to consume CP is not affected as more than 90% of the CP levels present in vivo was consumed during ischemia in both muscles. This suggests that the enzyme CK was not impaired as the muscles were able to consume a significant amount of CP under ischemic stress. The lower in vivo CP levels in the EDL due to estradiol and progesterone together can be attributed to an increased demand for CP, rather than a lower CK activity.

Theoretically, the ability to generate ATP during ischemia may be restricted when lower
in vivo glycogen and CP levels occur as these are the two key sources of ATP under this condition. This may be muscle type specific since the SOL muscle of females who received progesterone were energetically challenged, having lower in vivo ATP, as well as reduced glycogen. This may explain their lower net ATP consumption during ischemia. The SOL of progesterone females also had lower net glycogen consumption during ischemia, which would imply lower sources of ATP from glycolysis. These findings suggest that there are muscle fiber specific effects as to how energy levels are maintained, which may impact on ischemic energy levels.

Intriguingly, lactate which is the metabolic end product of anaerobic glycolysis was not affected by any of the sex hormones estradiol and progesterone in this study both in the EDL and SOL muscles. These data do not support what was hypothesized, whereby the predicted glycogen-sparing effect was supposed to reduce lactate accumulation during ischemia. What is particularly notable is that progesterone reduced glycogen sparing during ischemia in both the EDL and SOL, but there was no reduced lactate accumulation as a consequence of this glycogen sparing. Theoretically, when one glycosyl unit from glycogen is broken down, 2 units of lactate are produced during ischemia. As such, the fact that the net amount of lactate accumulated during ischemia was equal between the treatment groups in each muscle indicates that there are other sources for lactate, and not just that from glycogen during ischemia. This in fact is possible, as there are several precursor intermediates of glycolysis related to the enzymes measured (i.e. F-1,6-BP, pyruvate) that are present in the in vivo tissue prior to ischemia, which could account for the extra ischemic lactate produced. This is one limitation as this study did not measure those intermediate glycolytic components and they would have accumulated during ischemia in the different skeletal muscles.
Summary of Discussion for Study 2

Progesterone significantly reduced in vivo glycogen levels in slow-twitch muscles, but this effect was only achieved in the EDL when intermittent estradiol and progesterone were given together. This resulted in reduced glycogen consumption during ischemia in both muscles. Administration of both intermittent estradiol and progesterone reduced in vivo CP levels in both fast- and slow-twitch muscles. This resulted in reduced CP consumption during ischemia only in the fast-twitch muscles. In slow-twitch muscles, progesterone caused a small yet significant reduction in energy levels (ATP), which was removed when intermittent estradiol and progesterone were given together. Neither in vivo or ischemic lactate levels were altered by either the individual or combination administration of estradiol and progesterone. Since significant effects of estradiol and progesterone on in vivo and ischemic metabolic responses were observed, this work established the platform for the next series of experiments in Study 3, focusing on the role of the ER to determine if it has a role in mediating these hormone effects.

4.3. STUDY 3: The role of ER in skeletal muscle - effects on metabolism and its implication during ischemia in fast- and slow-twitch muscles

To further elucidate the potential mechanisms of the interaction of estradiol and progesterone on skeletal muscle metabolism, and to explore their effects as identified in Study 1 and 2, Study 3 examined the role of estrogen receptors (ER) since they are known to exist in skeletal muscle. Therefore, to clarify the role of the ER in these responses in different types of skeletal muscle, the ER antagonist ICI 182,780 (ICI) was used to investigate the down-regulation of the ER. Also, the selective ER modulator Raloxifene (RLX) was investigated as it has been shown to act as an ER agonist in skeletal muscle (Dieli-Conwright et al., 2009).
4.3.a. *In vivo* effects of ICI and RLX

The investigation of the role of ER in the metabolic function of skeletal muscles composed of different fiber types is a novel field of study, and the mechanisms by which estradiol or progesterone mediate skeletal muscle metabolism has yet to be clarified. To date, this is the first study of its kind to examine the role of ER on skeletal muscle CP, glycogen, lactate, and ATP levels in fast- and slow-twitch muscles. As reviewed in the Introduction, research into the metabolic role of ERs in skeletal muscle is a developing research topic. Skeletal muscle ER have been reported to exist in various species, including rats (Lemoine *et al.*, 2002; Dahlberg, 1982; Dionne *et al.*, 1979; Dube *et al.*, 1976) and humans (Lemoine *et al.*, 2003), and the specific roles of ER isoforms in glucose metabolism were identified. ERα positively modulates GLUT4 levels (Gorres *et al.*, 2011; Ribas *et al.*, 2010; Barros *et al.*, 2009). In the fast-twitch EDL, the ERα agonist propylpyrazoletriyl (PPT) increased GLUT4 levels, but not in the slow-twitch SOL (Gorres *et al.*, 2011). If ER isoforms regulate the entry of glucose into muscles, this would impact skeletal muscle glycogen levels.

The data in Study 3 suggested that there is a close relationship between the ER and skeletal muscle CP content. In OVX females, when the ER was blocked using the ER antagonist ICI 182,780, the data illustrated that *in vivo* CP levels were significantly reduced by 70 to 80% compared to OVX females in both slow- and fast-twitch muscles, respectively. In OVX females who received Raloxifene, CP values were similar to the OVX, yet 70-80% greater compared to ICI, in both muscles. This is a novel finding in skeletal muscle, but findings in other tissues suggests that there may be an association between estradiol and CP. For example, in malignant breast cancer tumours, an association between a high CP content and high ER content was reported, which the authors speculated could be attributed to the higher metabolic activity of this tissue (Barzilai *et al.*, 1991). Based on these observations and the data of Study 3, there is reason
to believe that there is a correlation between ERs and CP such that when the ER are blocked, CP content is reduced, and that this occurs in skeletal muscle independent of fiber type.

It was questioned whether the effect of ICI on in vivo CP levels in OVX females could be due to non-ovarian sources of estradiol. However, in rats, the enzyme 17alpha-hydroxylase/17,20 lyase (which participates in the conversion of pregnenolone and progesterone to dehydroepiandrosterone and androstenedione, respectively, see Figure 1.4) is not detected in the adrenal cortex (Pelletier et al., 2001), and the lack of this enzyme's activity has been reported by others for this tissue (Brock & Waterman, 1999; van Weerden et al., 1992). However, in contrast Zhao et al. (2005) reported that in OVX female rats, 17alpha-hydroxylase17,20 lyase mRNA was detected in the adrenal glands, albeit at lower levels, relative to intact females. Additionally, aromatase (which converts androstenedione and testosterone to estrone and estradiol, respectively, see Figure 1.4) has been shown to exist in skeletal muscle (Larionov et al., 2003). Based on this information, it can be speculated that there may be local production of estradiol in skeletal muscle. However, the levels of estradiol in the skeletal muscle tissue would need to be assessed in order to explore these theories and potentially explain how ICI decreased in vivo CP levels in OVX females which already have undetectable circulating estradiol (Appendix 3). It is postulated that if the estradiol produced locally in skeletal muscle has a regulatory role on in vivo CP levels, then ICI inhibited this estradiol effect, thereby resulting in significantly reduced in vivo CP levels in these females. The CP levels of the RLX matched that of OVX, indicating that RLX was not a significant ER agonist nor did it act as a ER blocker.

Although the work of Study 3 identified that in vivo CP was significantly reduced when the ER was inhibited by ICI and stayed at OVX levels when given Raloxifene, the data in Study 1 indicated that no significant effects of estradiol were observed on creatine kinase (CK) activity in the three fast-twitch muscles (gracilis, semitendinosus and EDL). It should be noted that in the
gracilis (muscle with least Type II fibers). OVX significantly lowered CK activity compared to intact females, and the mean CK activity of females given estradiol was 20% greater compared to OVX females. Recall that CK is the only enzyme that controls both the synthesis and breakdown of CP, which is a reversible reaction that converts CP and ADP to creatine and ATP (see Figure 1.2). The flux to sustain CP levels is a balance between CP production and consumption catalyzed by CK. In theory then, since inhibition of the ER with ICI resulted in ~80% lower CP levels, this would suggest two possibilities. Firstly, that CK activity was compromised, resulting in an inability to produce CP, or secondly, the demand for CP was significantly greater, resulting in lower CP levels. Prior to this thesis work, it was unknown what role ER would have on the regulation of CK in skeletal muscle. The findings of Study 2 were also the first to identify the role of intermittent estradiol and progesterone on CP levels in different types of skeletal muscles, and documented that progesterone decreased the amount of CP by 25% in both fast- and slow-twitch muscles. The mechanism for how progesterone mediated its effect on CP in skeletal muscle is unclear, but with the data from Study 3, the role of the ER is evident. It could be speculated that in Study 3, ICI impaired CK activity, and that this could have been accomplished by decreasing the synthesis of CK protein levels. As discussed in Study 1, genetic modifications in the expression of enzymes can regulate the activity of enzymes. In the case of CK, regulation by the ER has been reported. For example in breast cancer cells, estradiol reportedly stimulated the promotor region of one isoform of the CK gene which was later shown to contain an imperfect palindromic estrogen response element (ERE) that bound both ERα and other transcription factors (Wang et al., 2002). Thus, it could be speculated that in Study 3, CP levels in skeletal muscle were reduced by ICI because ICI down-regulated the expression of CK, resulting in reduced CK activity and hence reduced synthesis of CP in these muscles. Another potential explanation for the effects of ICI on in vivo CP could be related to the plasma
membrane ER, GPR-30. In contrast to ICI's antagonist effects on ER\(\alpha\) and ER\(\beta\), ICI has been recently reported to be an agonist of GPR-30 in reproductive and nervous tissue (Prossnitz & Maggiolini, 2009). GPR-30, nor its role in CP metabolism, has not yet been identified in either fast- or slow-twitch skeletal muscles. Should the presence of GPR-30 be confirmed and a connection with CP metabolism established in skeletal muscle, then ICI's agonistic activity on GPR-30 may have resulted in reduced in vivo CP levels observed in this study.

The thesis hypothesized that in skeletal muscle, the ER would play an important role in glycogen metabolism. This was based on strong evidence in the literature that illustrated significant metabolic properties in skeletal muscle attributed to estradiol, such as the glycogen-sparing effect during exercise (Hackney, 1999; Kendrick et al., 1987), increased glucose-uptake and glycogen storage (D'eon & Braun, 2002; Carrington & Bailey, 1985; Puah & Bailey, 1985; Ahmed-Sorour & Bailey, 1981), and also that the ER in skeletal muscle can regulate glucose metabolism (Gorres et al., 2011; Ribas et al., 2010; Barros et al., 2009). It was expected that since ER are present in skeletal muscle, ER affects glucose metabolism, and estradiol decreased the activity of GP and PFK activity (Study 1), it was hypothesized that the mechanism with which estradiol mediated changes in metabolism was via the ER and that glycogen metabolism would be sensitive to the ER modulators. It was proposed that this would occur to a greater extent in fast-twitch muscles compared to slow-twitch muscles since the enzyme data from Study 1 illustrated that estradiol decreased GP activity in muscles with a greater fast-twitch profile. The findings of Study 3 indicated that the ER does not regulate glycogen levels in skeletal muscle of either fast- or slow-twitch fiber type. Neither ICI nor RLX had any significant effect on in vivo glycogen levels in either fiber type. Unlike the results in the CP data, it can be concluded, based on the findings of Study 3, that blocking the ER does not augment glycogen levels in skeletal muscle.
4.3.b The effects of ICI and RLX on ischemic muscle metabolism

Modulating the ER had significant effects on ischemic CP consumption. The net CP consumption was significantly lower by ~90% in the EDL and ~80% in the SOL in ICI females compared to that of OVX and RLX females (Figure 3.3.2.), which is attributed to the lower amount of CP levels in these muscles. The percent CP consumption was also significantly lower in ICI females compared to OVX and RLX, whereby ~40% of available CP was used by ICI, compared to that of OVX and RLX which both used over 90% of available CP during ischemia. Thus, ICI impaired CP use during ischemia via the ER. CP consumption in RLX females matched that of OVX, supporting the finding that in skeletal muscle, RLX does not behave as an ER agonist on CP consumption. These finding support the claim that RLX does not act as a significant agonist on skeletal muscle CP use during ischemia, which was similarly observed with the in vivo CP data.

The ER findings also identified that they do not regulate ischemic glycogen responses, which is in contrast to what was hypothesized; this conclusion follows from the finding in Study 1 that glycogen phosphorylase (GP) was regulated by estradiol, which may explain estradiol’s glycogen-sparing effect described in the exercise literature. Unlike what was hypothesized, glycogen-sparing during ischemia due to the ER was not observed in Study 3. However, despite the lack of positive data, this information still provides a unique insight onto the role of the ER on skeletal muscle glycogen metabolism as the investigation into these issues in Study 3 are first of its kind and contribute new information on this topic. To date, it is unclear what other molecular mechanisms estradiol utilizes to alter glycogen metabolism.

Ischemic energy levels were significantly lowered by ICI compared to other groups, but the biological significance of these differences may be minor as the differences between the means were as low as 2% in some cases. Specifically, when looking at the absolute ischemic
ATP levels in the EDL, ICI females had a mean of 1.3 units of ATP versus RLX which had a mean of 0.9 units of ATP (Table 3.3.3), which was statistically significant; however, such ATP levels are very low and the difference could be considered biologically insignificant. Similarly, the net ATP consumption in the EDL of ICI females was ~4% lower compared to RLX females. ATP consumption during ischemia was not significantly reduced because in vivo ATP levels were not significantly changed by either ICI or RLX in both the EDL and SOL. Thus, despite ICI females having approximately 80-90% lower CP reserve prior to ischemia, ATP consumption was not affected. ATP consumption in RLX females matched that of OVX, confirming the finding that in skeletal muscle, RLX does not behave as an ER antagonist on ATP consumption. Again, these findings support the claim that RLX does not act as a significant agonist on skeletal muscle ATP use during ischemia, which was similarly observed for the in vivo ATP data.

Ischemic lactate profiles were not altered by ER manipulation in both muscle types. When combined with the results observed in Study 2, where estradiol did not have a significant effect on in vivo and ischemic lactate profiles, this finding implies that the ER does not alter anaerobic glycolysis in skeletal muscle irrespective of fiber type. Nevertheless, these observations are the first of their kind to be reported and illustrate that neither glycogen consumption (glycogenolysis) or lactate accumulation (anaerobic glycolysis) appear to be regulated by the ER.

Thus, the novel findings of Study 3 illustrate the importance of the ER in modulating CP levels in both fast- and slow-twitch skeletal muscles. The ER did not alter in vivo and ischemic glycogen metabolism, which did not alter anaerobic glycolysis (lactate levels). Although in vivo CP levels were lower in the presence of the ER antagonist ICI 182,780, energy levels (ATP) were not affected.
4.4. GENERAL DISCUSSION

The findings of this thesis have shed light on the metabolic adaptations that the sex hormones estradiol and progesterone exert on the regulation of the different components of glycogen and creatine phosphate metabolism in skeletal muscle with different fiber type properties. The implications of these metabolic responses were tested under ischemic conditions as they are key constituents involved in energy production under anaerobic conditions. Additionally, the significance of muscle fiber type was identified, confirming the importance of muscle fiber type composition when investigating the effects of these sex hormones on skeletal muscle metabolism. This work illustrated that; (a) muscle fiber types were important in determining the effect of estradiol and progesterone on glycogen and CP levels, (b) the ER regulated CP levels, and (c) the outcome of how glycogen and CP were consumed during ischemia were dependent on (a) and (b).

The muscle specific responses documented in Study 1 and Study 2 contribute new and valuable information with respect to the individual characteristics of skeletal muscles and how they respond to ovarian steroid hormones. Not all skeletal muscles are created equal, and their predisposed metabolic adaptations to meet their muscle fiber's appetite for energy must be taken into consideration when assessing sex hormone effects. To date, this is the first detailed investigation addressing these critical issues in skeletal muscle. Study 1 illustrated that even among fast-twitch muscles which are highly adapted for glycolytic metabolism, estradiol's impact on GP activity was not equal and that this was most likely associated with the distribution of Type I and II fibers. Specifically, estradiol decreased GP activity and this reduction occurred significantly in muscles with a high proportion of total type II fast-twitch fibers. Thus, it was concluded that in fast-twitch skeletal muscle, estradiol mediated its effects on glycogenolysis by controlling substrate use at the level of enzymatic activity (Figure 4.3) and that this is related to
the amount of total fast-twitch Type II fibers which are metabolically adapted to possess a
greater glycolytic potential compared to slow-twitch Type I fibers which have a lower glycolytic
potential. This work may explain the exercise literature's description of estradiol's 'glycogen-
sparing' effect observed in skeletal muscles during exercise in both women and in animal
models. PFK activity was also significantly regulated by estradiol in one fast-twitch muscle, and
not in the other two fast-twitch muscles. Reasons for this may be attributed to the expression of
transcription factors that regulated muscle metabolism in specific muscle types. It was speculated
that estradiol may down-regulate the transcription factor Nur77, which is expressed to a greater
degree in fast-twitch muscles compared to slow-twitch, resulting in decreased GP and PFK
activity (Figure 4.1). Thus, it was theorized that the variability in response to estradiol in the
different fast-twitch muscles was due to Nur77. Together with the above information, the
implication of these adaptations on metabolic potential may be important under conditions of
high glycolytic demand (such as ischemia) because glycolysis is a key source of energy.

The findings of Study 1 initiated the investigation into the role of intermittent estradiol
and progesterone on skeletal muscle metabolism and tested the muscle’s metabolic adaptations to
these hormones under ischemic conditions. This was undertaken in specific skeletal muscles that
were predominantly fast-twitch (EDL) and slow-twitch (SOL). Muscle specific effects of
progesterone on glycogen levels were also identified in Study 2. In the slow-twitch SOL muscle,
progesterone reduced \textit{in vivo} glycogen levels, which was sustained when intermittent estradiol
was given with progesterone (Figure 4.3). Interestingly this effect was not observed in the fast-
twitch EDL muscle; however, when progesterone was given in combination with intermittent
estradiol, \textit{in vivo} glycogen and CP levels were reduced in the EDL, suggesting that in fast-twitch
muscles, intermittent estradiol is required to elicit progesterone effects. These data led to two key
thoughts, firstly how does progesterone acts in skeletal muscle, and secondly, how does this
explain muscle fiber specific effects. To date, the mechanism(s) by which progesterone mediates changes in glycogen metabolism in skeletal muscle remains unknown. In the literature, authors have speculated that PGRMC-1 may be involved as its levels are greater in slow-twitch muscles compared to fast-twitch muscles. It was thus speculated that PGRMC-1 may be associated with mediating the effects of progesterone on glycogen metabolism. However, it is unknown whether other hormones, such as estradiol, also stimulate PGRMC-1 expression and whether this could explain the muscle fiber specific responses discovered in Study 2.

The implications of the effects of estradiol and progesterone, and the ER role in skeletal muscle metabolic responses were investigated under ischemic stress. Overall, the data revealed that both intermittent estradiol and progesterone reduced glycogen and CP consumption during ischemia due to lower \textit{in vivo} levels of glycogen and CP, respectively, and that muscle dependent effects were observed. Specifically, the EDL required both intermittent estradiol and progesterone to elicit glycogen-sparing during ischemia, whereas in the SOL, this effect was observed with progesterone alone and sustained when both hormones were given. The percent glycogen consumption was not altered by progesterone (given with or without intermittent estradiol) suggesting that progesterone did not inhibit the capacity of the muscles to utilize glycogen, but only inhibited \textit{in vivo} glycogen levels, proving that progesterone mediates its glycogen-sparing effect by regulating substrate levels. This information is especially important to the exercise literature which to date has only acknowledged estradiol's ability to spare glycogen. This thesis has identified that progesterone is also a valid concern. Unlike what was hypothesized, the data illustrated that the ER was not involved mediating a significant effect on glycogen metabolism in either fast- or slow-twitch muscles, suggesting that the relation between intermittent estradiol and progesterone on glycogen consumption is not ER driven.

CP metabolism in skeletal muscle is significantly regulated by estradiol and progesterone,
and the ER may be involved. The finding of Study 2 indicated that when both intermittent estradiol and progesterone were given together, significant reductions in CP levels were observed, and intriguingly, this observation was conserved in both fast- and slow-twitch muscles (Figure 4.3). The findings of Study 3 support that the mechanism for this is via the ER, since blocking the ER with ICI resulted in a 70-80% reduction in CP levels, which was also observed in both slow- and fast-twitch muscles, respectively (Figure 4.3). During ischemia, CP consumption was reduced by intermittent estradiol and progesterone, but only in the EDL which may be due to this muscle's 40% greater amount of CP compared to the SOL. The capacity to utilize CP was not altered by estradiol and progesterone as the percent CP consumption illustrated that more than 90% of the CP was consumed during ischemia in both muscle types.

Overall, this doctoral work has demonstrated that it is critical to identify the role of skeletal muscle fiber type composition when investigating the role of estradiol and progesterone on muscle metabolism, even among different fast-twitch muscles. Specifically, the implications of these metabolic effects were investigated using an ischemic model which identified that estradiol and progesterone caused glycogen-sparing for separate reasons; estradiol reduced the activity of the enzyme GP which breaks down glycogen, whereas progesterone reduced the amount of glycogen available for glycogenolysis. This was muscle fiber specific as muscles with greater fast-twitch fiber composition illustrated estradiol's effect on GP, whereas those of progesterone are greater in slow-twitch muscles. This doctoral work also demonstrated that the ER antagonist ICI impaired CP levels, highlighting the importance of skeletal muscle ER on CP metabolism.
Figure 4.3. Summary of significant findings of thesis work illustrating the effects of estradiol (E), progesterone (P), and ICI on *in vivo* muscle metabolism. Muscle fiber effects are indicated in text along arrow lines. E decreased GP and PFK activity in muscles with greatest percentage fast-twitch fibers. P alone decreased glycogen levels in slow-twitch muscles and decreased CP levels in fast-twitch muscles, and this effect was sustained when also given with intermittent-estradiol (IE). Glycogen levels were decreased in fast-twitch muscles and CP levels were decreased in slow-twitch muscles only when IE+P were given together. ICI down-regulation of the ER significantly decreased CP levels in both muscle types. Implications of these metabolic changes were studied under ischemia (not shown in figure). E and P resulted in glycogen-sparing during ischemia for two different reasons: (1) P reduced availability of glycogen as a substrate; (2) whereas E decreased the activity of GP which breaks-down glycogen. Mitochondrial enzymes were unaffected by E.
4.5 CONCLUSIONS

1. Estradiol decreased the activity of the key enzymes involved in glycogenolysis (glycogen phosphorylase) and glycolysis (phosphofructokinase), but not anaerobic glycolysis (lactate dehydrogenase), and this was greater in skeletal muscles with a higher fast-twitch composition. Estradiol did not affect aerobic enzymes in any of the fast-twitch muscles studied.

2. a) Estradiol did not increase in vivo glycogen and CP levels to a greater extent in fast-twitch muscles compared to slow-twitch muscles. Blocking the ER resulted in decreased in vivo CP levels in both fast- and slow-twitch muscles.

b) During ischemia, estradiol did not decrease anaerobic glycolysis (lactate accumulation) and energy levels (ATP) because of decreased glycogenolysis due to a lowered glycogen phosphorylase activity. The ER did not have a regulatory role in either muscle type.

3. a) Progesterone decreased in vivo glycogen levels in slow-twitch muscles compared to fast-twitch muscles.

b) During ischemia, progesterone decreased glycogen consumption as a result of decreased glycogen availability, but this did not affect either anaerobic glycolysis (lactate accumulation), or energy levels (ATP, CP), and was observed in slow-twitch muscles and not fast-twitch muscles.
4. Progesterone did not inhibit the metabolic effects of estradiol when given in combination in either muscle types.
4.6 LIMITATIONS

(1) The maximal enzymatic activity of each enzyme was assessed using *in vitro* techniques in these fast-twitch muscles, which may not accurately reflect *in vivo* conditions. (STUDY 1)

(2) Progesterone could have been studied to determine what role it may have on these enzyme activities, especially in instances where estradiol was shown not to return enzyme activities back to gonadally intact female levels. (STUDY 1)

(3) The model of ischemia used in Study 2 and 3 was three hours long, and was selected to maximize the metabolic ischemic responses without comprising tissue viability. It is possible that some of the glycogenolytic responses during ischemia may occur to a greater degree at earlier ischemic time points and future work could examine this.

(4) Other metabolic variables could have been measured during ischemia in Study 2 and 3 to identify where along the glycolytic pathway other sources of lactate were being generated. For instance, the levels of F-1,6-BP and pyruvate could have been measured to identify this.

(5) Although energy sources were completely utilized by the end of ischemia, other breakdown products of ATP, such as ADP, AMP, and the breakdown products of AMP, could have been measured to determine the impact of estradiol and progesterone on total adenine nucleotide (TAN) content of each muscle in Study 2 and 3.

(6) The SERM Raloxifene was selected based on its publicized ability to act as an agonist in other tissues (bone, heart). It was assumed that Raloxifene would also act as an agonist in
skeletal muscle; however, it will be necessary for future work to confirm whether Raloxifene always behaves as an agonist or an antagonist in skeletal muscle. Other SERMs could have also been studied and compared to Raloxifene to clarify their role in skeletal muscle metabolism.

(7) ERα and ERβ levels could have been measured to determine the impact of the different ER modulators on their expression and associate them with changes in in vivo CP levels.

(8) The model of intermittent estradiol administration in Study 2 impaired the ability to investigate physiologic levels of estradiol on the measured metabolic parameters of skeletal muscle metabolism.
4.7 FUTURE DIRECTIONS

The findings of this thesis work have generated new queries for future studies in the area of muscle metabolism, sex hormones, and the mechanisms which mediate the sex hormone effects in skeletal muscle. In addition, several limitations to this thesis work were also identified which warrant further investigation. This section will review potential future directions of this work.

To date, the role of progesterone in mediating sex differences in muscle metabolism has not received the same degree of investigation as estradiol in the literature. The work described in this thesis identified significant effects of progesterone on both glycogen and creatine phosphate metabolism that was skeletal muscle fiber type specific. Future work would focus on the mechanisms with which progesterone mediates its effects in skeletal muscle since little is known. Several ways to explore how progesterone acts in skeletal are proposed. Nuclear hormone action in a target cell has been described to occur via nuclear hormone receptors which, upon binding to a hormone, mediate changes in gene expression in the nucleus. Studies investigating progesterone receptor (PR) properties would determine if this 'classical' mechanism of progesterone action exists in skeletal muscle. Currently, the literature is unclear regarding the presence of skeletal muscle PR, which if any, PR isoforms are present in skeletal muscle, and whether there are fiber type differences in PR isoform expression. Thus, these areas would be clarified by measuring mRNA and protein levels of each PR isoform (A and B) in fast- and slow-twitch skeletal muscles. The muscle specific effects could be further explored by assessing myosin heavy chain (MHC) isoform expression and determine if there is a correlation between PR isoforms and MHC isoforms.

To further clarify how various metabolic enzymes may be responsible for the hormone effects identified, future work would also clarify the role of progesterone, and its interaction with
estradiol, on the activity of metabolic enzymes, including identifying muscle fiber specific
effects by cross comparing slow-twitch (SOL) to fast-twitch muscles (EDL). For example, the
activity of glycogen synthase could also be measured because (a) it is a key regulator of
glycogen synthesis, and (b) it may explain the reduced in vivo glycogen levels due to
progesterone in skeletal muscle (Study 2). Since the classical mechanism of nuclear hormone
action of progesterone is being proposed, experiments measuring the expression (mRNA and
protein levels) of key regulatory enzymes that are regulated by progesterone and estradiol could
be measured to support or refute this theory. Determining the MHC isoform expression would
clarify muscle specific effects in these studies examining how estradiol and progesterone
modulate the activity of metabolic enzymes.

The mechanism with which estradiol mediates its effects on skeletal muscle metabolism
was explored in this thesis. The work using ICI as an ER blocker to study ER effects on skeletal
muscle metabolism is novel, and these data gave some insight into the process with which ER
modulate CP metabolism. Future experiments could determine which isoform of the ER is
responsible for reducing CP levels in both skeletal muscle fiber types. This could be studied by
using ERα and ERβ knockout (KO) mice models, whereby in vivo CP and CK activity levels
could be measured in fast- and slow-twitch skeletal muscles. Or, an ERα antagonist such as
methyl-piperidino-pyrazole dichloride (MPP) and ERβ antagonist 4-[2-Phenyl-5,7-
bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), could be studied to
determine which of these ER isoforms are responsible for the effects observed above. The above
work can also be extended to study the effect that specific ER isoform antagonists have on the
profile of specific metabolic enzymes in different muscle types.

Identifying the role of the various transcriptions factors responsible for the metabolic
differentiation of fast- and slow-twitch muscles would further our knowledge as to how estradiol
and progesterone might cause muscle fiber specific effects. One example is the nuclear orphan transcription factor Nur77, which is expressed to a greater extent in fast-twitch muscles compared to slow-twitch muscles and up-regulates glucose metabolism. If Nur77 mRNA and protein content is assessed in various skeletal muscle types, it may explain why estradiol alters GP and PFK activity among the fast-twitch muscles of Study 1. For example, the relationship between Nur77 expression and the activity of metabolic enzymes, such as GP and PFK, in each muscle could be correlated to confirm their role and further demonstrate the theory proposed in Figure 4.1. If confirmed, this could be further explored using a Nur77 KO mice model, and the activity and expression of various metabolic enzymes, including glycogen levels, could be measured in fast- and slow-twitch muscles. Other transcription factors which regulate the metabolic differentiation of fast- and slow-twitch muscles include the receptor interacting protein 140 (RIP140). This is a transcriptional corepressor expressed to a greater extent in muscles with a greater glycolytic potential (fast-twitch) compared to those with a lower one (slow-twitch). In mice lacking RIP140 gene, genes participating in fatty acid oxidation and oxidative phosphorylation are up-regulated. RIP140 can interact with the ER, but what impact this interaction might have on skeletal muscle metabolism is currently unknown and could be investigated to explain the muscle specific responses of estradiol and progesterone between the EDL and SOL. Another transcription factor that could be investigated is the peroxisome proliferator activated receptor γ co-activator 1α (PGC1α) which regulates energy metabolism and mitochondrial biogenesis in skeletal muscle with a high oxidative potential (slow-twitch, Type IIa fast-twitch fibers). What role estradiol or progesterone have on PGC1α expression in skeletal muscle of different fiber types is not known. In other tissues, PGC1α expression was decreased by estradiol in both the uterus and brain blood vessels. Whether this occurs differentially in fast- and slow-twitch skeletal muscles is currently unknown, and could be
studied to further elucidate the role of estradiol and progesterone in different types of skeletal muscle.

In summary, the proposed areas of investigations would help to clarify the mechanisms with which progesterone and estradiol mediate changes in various types of skeletal muscle. Using these various experimental models, this work would expand our understanding of how these hormones, along with their receptors, participate in metabolism and potentially explain further the findings of this PhD work.
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APPENDIX 1: Muscle Fiber Distribution of Various Skeletal Muscles Referenced in Thesis

This appendix will summarize the fiber type content of the skeletal muscles utilized in the three experimental studies of this thesis, and also of those skeletal muscles that were reviewed in the Introduction. Please note that the data to identify the proportion of the slow-twitch Type I fibers and the fast-twitch Type IIa, Type IID/X, and Type IIb fibers in these skeletal muscle was taken from a paper published by Delp & Duan (1996) which did a thorough overview to quantify all the different fiber populations in rat skeletal muscles.

Appendix Table 1 identifies the proportion of slow- and fast-twitch fibers of the skeletal muscles utilized in this thesis. In Study 1, the fast-twitch gracilis (GRAC), semitendinosus (SMT), and extensor digitorum longus (EDL) muscles were studied. In Study 2 and 3, the slow-twitch soleus (SOL) and fast-twitch EDL were studied. To further analyze these data, the sum of the means for the total % Type II fibers (Type IIa + Type IID/X + Type IIb) was compared to the total % Type I. Additionally, the CS activity reported by Delp & Duan (1996) was also included to illustrate the differences in oxidative potential among the differing muscles.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>% Type I</th>
<th>% Type IIa</th>
<th>% Type IID/X</th>
<th>% Type IIb</th>
<th>Total % Type II</th>
<th>CS activity (µmol/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRAC</td>
<td>20 ± 15</td>
<td>3 ± 2</td>
<td>6 ± 3</td>
<td>71 ± 18</td>
<td>~80</td>
<td>15.4 ± 1.1</td>
</tr>
<tr>
<td>SMT</td>
<td>6 ± 2</td>
<td>11 ± 2</td>
<td>24 ± 4</td>
<td>59 ± 6</td>
<td>~94</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>EDL</td>
<td>4 ± 1</td>
<td>20 ± 3</td>
<td>38 ± 3</td>
<td>38 ± 3</td>
<td>~96</td>
<td>21.6 ± 2.1</td>
</tr>
<tr>
<td>SOL</td>
<td>84 ± 4</td>
<td>7 ± 0</td>
<td>9 ± 6</td>
<td>0</td>
<td>~15</td>
<td>21.3 ± 2.3</td>
</tr>
</tbody>
</table>

Appendix Table 1.
As illustrated in Appendix Table 1, the total % Type II fibers were the greatest in the EDL, followed by the SMT, the GRAC, and the least amount in the SOL. The distribution of the various isoforms of Type II fibers also varied among the fast-twitch muscles (indicated in blue rows) and slow-twitch muscles (indicated in pink row).

Appendix Table 2 identifies the proportion of slow- and fast-twitch fibers in various fast-twitch muscles that were reviewed in the Introduction.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>% type 1</th>
<th>% type 2a</th>
<th>% type2D/X</th>
<th>% type 2b</th>
<th>Total % Type II</th>
<th>CS activity (µmol/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vastus lateralis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>red</td>
<td>16 ± 6</td>
<td>33 ± 4</td>
<td>32 ± 3</td>
<td>19 ± 10</td>
<td>~84</td>
<td>42.3 ± 3.1</td>
</tr>
<tr>
<td>white</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100 ± 0</td>
<td>100</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>vastus medialis</td>
<td>8 ± 6</td>
<td>10 ± 9</td>
<td>20 ± 2</td>
<td>62 ± 17</td>
<td>~92</td>
<td>20.2 ± 1.4</td>
</tr>
<tr>
<td>gastrocnemius</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>red</td>
<td>51 ± 4</td>
<td>35 ± 3</td>
<td>13 ± 7</td>
<td>1 ± 1</td>
<td>~49</td>
<td>36.2 ± 1.6</td>
</tr>
<tr>
<td>white</td>
<td>0</td>
<td>0</td>
<td>8 ± 4</td>
<td>92 ± 4</td>
<td>100</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>tibialis anterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>red</td>
<td>7 ± 2</td>
<td>30 ± 3</td>
<td>37 ± 7</td>
<td>26 ± 1</td>
<td>~93</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>white</td>
<td>2 ± 1</td>
<td>18 ± 5</td>
<td>34 ± 2</td>
<td>46 ± 8</td>
<td>~98</td>
<td>18 ± 0.9</td>
</tr>
<tr>
<td>plantaris</td>
<td>6 ± 2</td>
<td>14 ± 2</td>
<td>33 ± 2</td>
<td>47 ± 2</td>
<td>~94</td>
<td>22 ± 1</td>
</tr>
</tbody>
</table>

Appendix Table 2.
APPENDIX 2: Uterus to Body Weight Ratios of Experimental Animals of Studies 1, 2, 3

For each rat utilized in the experimental studies of this thesis, the body weight (bw) and uterus weights (uw) were measured at the termination of the experiments. Rats were weighed prior to anesthesia, and the uterus was excised post-mortem. Weights were recorded and the uterus weight ratio was calculated as follows: UW:BW Ratio = UW/BW. This ratio was calculated to determine the effect of OVX and estradiol treatment and correct for differences in body weight and uterus weights in these experimental groups.

STUDY 1

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>INT</th>
<th>OVX</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>300 ± 18</td>
<td>409 ± 22 *</td>
<td>236 ± 16 * **</td>
</tr>
<tr>
<td>Uterus Weight (x10^-3 g)</td>
<td>680 ± 245</td>
<td>27 ± 8 *</td>
<td>332 ± 43</td>
</tr>
<tr>
<td>Uterus Weight: Body Weight Ratio (x10^-3)</td>
<td>2.2 ± 0.7</td>
<td>0.07 ± 0.02 *</td>
<td>1.4 ± 0.3 *</td>
</tr>
</tbody>
</table>

Appendix Table 3. Body weight, uterus weight, uterus:body weight ratios of sham-operated (INT), OVX, and ET Sprague Dawley females. Values expressed as mean ± SD. BW data analyzed using one-way ANOVA with Bonferroni post-hoc, UW and UW:BW data analyzed using a Kruskal-Wallis one-way ANOVA on Ranks. * p<0.05 vs Intact, ** p<0.05 vs OVX.

Body weight of OVX females in Study 1 was significantly greater by 27% in INT and 42% in ET females (Appendix Table 3). Body weight of ET females was significantly lower by 21% compared to INT females (Appendix Table 3).

Uterus weight of OVX females was significantly lower by over 90% compared to INT
females. Although ET females had 90% greater uterus weights compared to OVX, this was not statistically significant.

UW:BW ratios of INT females were significantly lower by 97% in OVX and 37% in ET females. Although ET females had 95% greater UW:BW ratios compared to OVX, this was not statistically significant.

**STUDY 2**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>OVX</th>
<th>IE</th>
<th>P</th>
<th>IE+P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>443 ± 60</td>
<td>379 ± 38</td>
<td>422 ± 39</td>
<td>340 ± 51 <em>,</em>*</td>
</tr>
<tr>
<td><strong>Uterus Weight (x10^-3 g)</strong></td>
<td>28 ± 4</td>
<td>251 ± 107 <em>,</em>*</td>
<td>40 ± 4</td>
<td>222 ± 163 *</td>
</tr>
<tr>
<td><strong>Uterus Weight: Body Weight Ratio (x10^-3)</strong></td>
<td>0.06 ± 0.01</td>
<td>0.7 ± 0.3 <em>,</em>*</td>
<td>0.1 ± 0.02</td>
<td>0.7 ± 0.5 <em>,</em>*</td>
</tr>
</tbody>
</table>

**Appendix Table 4.** Body weight, uterus weight, uterus:body weight ratios of OVX, IE, P, and IE+P Sprague Dawley females. Values expressed as mean ± SD. UW data analyzed using one-way ANOVA with Bonferroni post-hoc, BW and UW:BW data analyzed using a Kruskal-Wallis one-way ANOVA on Ranks. * p<0.05 vs OVX, ** p<0.05 vs P.

Body weight in Study 2 was not significantly different among OVX, IE, and P females (Appendix Table 4). Body weight of IE+P females was significantly lower by 23% in OVX and 19% in P females.

Uterus weights of IE and IE+P females were approximately 89% greater compared to OVX females.

UW:BW ratio of IE and IE+P females were approximately 91% greater compared to
OVX females. UW: BW ratios of OVX and P females were not statistically different from each other. UW: BW ratios of IE and IE+P were not statistically different from each other.

**STUDY 3**

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<tr>
<th>Experimental Group</th>
<th>OVX</th>
<th>ICI</th>
<th>RLX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>443 ± 60</td>
<td>417 ± 46</td>
<td>334 ± 13 *</td>
</tr>
<tr>
<td><strong>Uterus Weight (x10^-3 g)</strong></td>
<td>28 ± 4</td>
<td>33 ± 7</td>
<td>66 ± 10 *</td>
</tr>
<tr>
<td><strong>Uterus Weight: Body Weight Ratio (x10^-3)</strong></td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.2 ± 0.03 <em>,</em>*</td>
</tr>
</tbody>
</table>

*Appendix Table 5.* Body weight, uterus weight, uterus:body weight ratios of OVX, ICI, and RLX Sprague Dawley females. Values expressed as mean ± SD. BW data analyzed using one-way ANOVA with Bonferroni post-hoc, UW and UW:BW data analyzed using a Kruskal-Wallis one-way ANOVA on Ranks. * p<0.05 vs OVX, ** p<0.05 vs ICI.

Body weight in Study 3 of RLX females was 25% lower compared to OVX females. Body weight of ICI and RLX females were not statistically different from each other (Appendix Table 5).

Uterus weight of RLX females was 58% greater compared to OVX females. Uterus weight of ICI and RLX females were not statistically different from each other.

UW:BW ratio of RLX females were significantly greater by 70% in OVX and 61% in ICI females. UW:BW ratio of ICI and RLX females were not statistically different from each other. UW:BW ratio of OVX and ICI females were not statistically different from each other.
APPENDIX 3: Plasma 17β-Estradiol Levels in Experimental Animals of Studies 1, 2, 3

Plasma 17β-estradiol levels of each female in each study were assessed via RIA assay technique as described in Chapter 2 of the Methods section. The term 'low' was reported by the gamma counter whenever the value being read was below the RIA assay's lower limit to assess the minimal amount of 17β-estradiol in the plasma, which was < 8 pg/ml (not detectable).

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Plasma 17β-estradiol (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STUDY 1</strong></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>not detectable</td>
</tr>
<tr>
<td>OVX</td>
<td>not detectable</td>
</tr>
<tr>
<td>ET</td>
<td>153.6 ± 55.2</td>
</tr>
<tr>
<td><strong>STUDY 2</strong></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>not detectable</td>
</tr>
<tr>
<td>IE</td>
<td>not detectable</td>
</tr>
<tr>
<td>P</td>
<td>not detectable</td>
</tr>
<tr>
<td>IE+P</td>
<td>not detectable</td>
</tr>
<tr>
<td><strong>STUDY 3</strong></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>not detectable</td>
</tr>
<tr>
<td>ICI</td>
<td>not detectable</td>
</tr>
<tr>
<td>RLX</td>
<td>not detectable</td>
</tr>
</tbody>
</table>

**Appendix Table 6.** Plasma estradiol levels (pg/ml) reported in female Sprague-Dawley rats measured at the end of 120 days of treatment.
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