2016

Nutritional Modulation of Reproductive Potential in Dairy Bulls

Dance, Alysha

http://hdl.handle.net/11023/3054
doctoral thesis

University of Calgary graduate students retain copyright ownership and moral rights for their thesis. You may use this material in any way that is permitted by the Copyright Act or through licensing that has been assigned to the document. For uses that are not allowable under copyright legislation or licensing, you are required to seek permission.

Downloaded from PRISM: https://prism.ucalgary.ca
Nutritional Modulation of Reproductive Potential in Dairy Bulls

by

Alysha Lynne Dance

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

JUNE, 2016

© Alysha Lynne Dance 2016
Abstract

Holstein bull calves often reach AI centers in suboptimal body condition. Early-life nutrition is reported to increase reproductive performance in beef bulls. The general aim of this thesis was to determine effects of early-life nutrition on reproductive performance of Holstein bulls. We hypothesized that Holstein bull calves on high-nutrition diets during early-life will have larger testes, greater sperm production, and be younger at puberty than those on low-nutrition diets. We determined that bulls fed the high-nutrition diet from 2 to 31 wk were younger at puberty, had larger testes and had higher concentrations of circulating IGF-I than low-nutrition bulls. Furthermore, early-life nutritional modulation did not alter semen quality. The high-nutrition bulls had more proliferating and differentiating Sertoli cells earlier in life than low-nutrition bulls and IGF-I promoted proliferation of bovine Sertoli cells in culture. Overall we demonstrated that IGF-I has important roles in bovine reproduction and we identified key periods during bull development where it appeared that nutritional modulation affected testes development. Therefore, nutritional modulation can be recommended as a management tool to improve reproductive potential of AI bulls. In addition, we inferred that nutritional modulation may serve as a model for studying mechanisms controlling reproductive development in bulls.
Acknowledgements

First and foremost, I thank and extend my appreciation to my supervisors Drs. Jacob Thundathil and John Kastelic. Your support, guidance and leadership have been invaluable to my development as a researcher. When I started as a Master’s student 5.5 years ago, I never imaged completing my doctorate and with your tutelage, this journey has been possible.

Additionally, I thank and acknowledge my committee members for their time, effort, and guidance over the last 5.5 years: Drs. Ina Dobrinski, Claudia Klein and Frans van der Hoorn. Dr. Klein, thank you for the extra meetings, technical support, and use of your laboratory.

I owe a special thank you to all the people who helped with the animal work associated with the Lethbridge portion of this project: Randy Wilde, your help was invaluable as I started out and for taking so much extra time to help me learn the ropes and always being available for my questions; the staff of the Lethbridge Research Centre who cared for the calves and helped us on sampling days; and the staff at Ben’s Quality Meats for allowing us to collect all of our samples as we wanted on the kill floor; and Patrick Blondin and his colleagues at L’Alliance Bovitech Inc for their project oversight and technical support.

In addition, there were many other individuals involved in the animal work, done in Strathmore. Thank you to: Geritt Van Hierden for feeding the milk diets and allowing us to work on your farm; William Torres, Mick Taylor, and Kristine Burgess at Cattleland Feedyards Ltd., I thank you for your help with managing the calves at Cattleland and for going above and beyond to help with the feeding; Arianna Dirk, your help feeding calves for the summer allowed me to be at the university, completing necessary lab work; and to the Beiseker and Neudorf Hutterite colonies for supplying bull testes to optimize my culture experiments.
Special thank you and acknowledgement to the financial supporters of this work: The Canadian Agricultural Adaptation Program, L’Alliance Bovitech Inc., University of Calgary Margaret Gunn endowment for animal research, and UCVM’s Eye’s High funding program.

I thank the past and present members of the Thundathil laboratory who have helped me in my research in one way or another: Dr. Ajitkumar Menon, Dr. Habib Shojaei, Dr. Gayathri Rajamanickam, Dr. Sulochana Krishnakumar, Doug Nickel, Dr. Diogo Camara, Dr. Karina Goularte, Dr. Chinju Johnson, Dr. Guilherme Rizzoto, and Mina Ojaghi. Additionally, I want to thank the members of the other three labs on the 4th floor of HMRB (Drs. Dobrinski, van der Hoorn and Biernaskie lab’s) who have helped in many ways over the years.

Thank you to my friends, including those that I made while in grad school, for humoring me through the ups and downs of research and keeping me sane.
Dedication

To my husband Stuart, for being my best friend, confidant and biggest fan.

This would not have been possible without you

and

to my parents, Tom and Nancy, for the unwavering support and for the encouragement to

follow my dreams.
Table of Contents

Abstract ............................................................................................................................... ii
Acknowledgements ............................................................................................................ iii
Dedication ............................................................................................................................v
Table of Contents ............................................................................................................... vi
List of Tables ..................................................................................................................... ix
List of Figures ......................................................................................................................x
List of Abbreviations ....................................................................................................... xiii

CHAPTER ONE: INTRODUCTION .......................................................................1
  1.1 Canadian artificial insemination (AI) industry ..........................................................1
  1.2 Reproductive development of bulls .........................................................................3
    1.2.1 Endocrine changes during reproductive development of the bull .................3
    1.2.2 Testicular changes during reproductive development of the bull ...............5
      1.2.2.1 Leydig cell development .................................................................6
      1.2.2.2 Sertoli cell development .........................................................8
      1.2.2.3 Germ cell development .........................................................10
    1.2.3 Puberty .........................................................................................11
    1.2.4 Hastening puberty through endocrine manipulation ..................................13
  1.3 Links between nutrition and reproduction .............................................................14
    1.3.1 Insulin-like growth factor-I (IGF-I) .........................................................16
      1.3.1.1 IGF-I signaling ............................................................................17
      1.3.1.2 IGF-I and reproduction .............................................................20
      1.3.1.3 IGF-I and Leydig cells .............................................................21
      1.3.1.4 IGF-I and Sertoli cells .............................................................22
  1.4 Aims, hypotheses, objectives, and highlights .........................................................24

CHAPTER TWO: ENHANCED EARLY-LIFE NUTRITION PROMOTES HORMONE PRODUCTION AND REPRODUCTIVE DEVELOPMENT IN HOLSTEIN BULLS .................................................................................................................... 29
  2.1 Preamble ..................................................................................................................29
  2.2 Abstract ....................................................................................................................29
  2.3 Introduction ..............................................................................................................31
  2.4 Materials and methods ...........................................................................................33
    2.4.1 Bulls and treatments .................................................................................33
    2.4.2 Testicular characteristics ...........................................................................34
    2.4.3 Blood samples and hormone analyses .......................................................35
    2.4.4 Age at puberty ............................................................................................37
    2.4.5 Seminiferous tubular diameter .................................................................37
    2.4.6 Testicular gene expression .........................................................................38
      2.4.6.1 Isolation of RNA ............................................................................38
      2.4.6.2 Real-Time RT-PCR .........................................................................38
    2.4.7 Statistical analyses .......................................................................................39
  2.5 Results ......................................................................................................................40
  2.6 Discussion ................................................................................................................55
  2.7 Acknowledgements .................................................................................................61
CHAPTER THREE: ENHANCED EARLY-LIFE NUTRITION OF HOLSTEIN BULLS INCREASES SPERM PRODUCTION POTENTIAL WITHOUT DECREASING POST-PUBERTAL SEMEN QUALITY ........................................................ 62
  3.1 Preamble ..................................................................................................................62
  3.2 Abstract ....................................................................................................................62
  3.3 Introduction ..............................................................................................................64
  3.4 Materials and methods .............................................................................................65
    3.4.1 Bulls and treatments ........................................................................................65
    3.4.2 Cost of differential feeding ..............................................................................66
    3.4.3 Sexual development and testicular characteristics ..........................................68
    3.4.4 Age at maturity ................................................................................................68
    3.4.5 Incremental value of increased sperm production ...........................................69
    3.4.6 Semen freezing ................................................................................................70
    3.4.7 Evaluation of motion characteristics of post-thaw sperm ...............................71
    3.4.8 Evaluation of in vitro fertilizing ability ...........................................................71
    3.4.9 Sperm viability ................................................................................................73
    3.4.10 Two-dimensional (2D) gel electrophoresis (sperm proteins) ........................75
    3.4.11 Protein profile analysis ..................................................................................75
    3.4.12 Statistical analyses .........................................................................................76
  3.5 Results ......................................................................................................................76
  3.6 Discussion ................................................................................................................83
  3.7 Acknowledgements ..................................................................................................88

CHAPTER FOUR: HIGHER EARLY-LIFE NUTRITION INCREASES PROLIFERATION AND MATURATION OF SERTOLI CELLS AT 24 WK OF AGE IN HOLSTEIN BULL CALVES ............................................................ 89
  4.1 Abstract ....................................................................................................................89
  4.2 Introduction ..............................................................................................................89
  4.3 Materials and methods .............................................................................................91
    4.3.1 Bulls and treatments ........................................................................................91
    4.3.2 Castrations .......................................................................................................92
    4.3.3 Tubular diameter .............................................................................................93
    4.3.4 Testicular gene expression ..............................................................................93
      4.3.4.1 Isolation of RNA. ...................................................................................93
      4.3.4.2 Real-Time RT-PCR ...............................................................................93
    4.3.5 Immunohistochemistry ....................................................................................94
    4.3.6 Statistical analysis ...........................................................................................95
  4.4 Results ......................................................................................................................96
    4.4.1 Body weight and testes size .............................................................................96
    4.4.2 Tubular diameter .............................................................................................97
    4.4.3 Mature Sertoli cell markers .............................................................................99
    4.4.4 Immature Sertoli cell markers .........................................................................99
    4.4.5 Proliferation markers .....................................................................................100
    4.4.6 IGF-I markers ................................................................................................105
  4.5 Discussion ..............................................................................................................106
  4.6 Acknowledgments .................................................................................................110
CHAPTER FIVE: A COMBINATION OF IGF-I AND FSH PROMOTES PROLIFERATION OF PREPUBERTAL BOVINE SERTOLI CELLS ISOLATED AND CULTURED IN VITRO ......................................................... 111

5.1 Preamble .................................................................................................................................. 111
5.2 Abstract .................................................................................................................................... 111
5.3 Introduction ............................................................................................................................... 112
5.4 Materials and methods ............................................................................................................. 114
   5.4.1 Bulls ................................................................................................................................. 114
   5.4.2 Cell isolation .................................................................................................................... 115
   5.4.3 Sertoli cell number and viability ...................................................................................... 119
   5.4.4 Analyses of purity of Sertoli cells by immunofluorescence microscopy .......................... 119
   5.4.5 Protein extraction and western blotting .............................................................. 120
   5.4.6 Hormone treatments ....................................................................................................... 120
   5.4.7 Evaluating cell number ................................................................................................... 121
   5.4.8 Evaluating cell proliferation ......................................................................................... 121
   5.4.9 Statistical analyses ........................................................................................................... 121
5.5 Results ...................................................................................................................................... 122
   5.5.1 Sertoli cell number and viability ..................................................................................... 122
   5.5.2 Purity ................................................................................................................................... 124
   5.5.3 Cell numbers after IGF-I treatment .............................................................................. 125
   5.5.4 Percentage of Sertoli cells expressing proliferation marker in response to IGF-I treatment ........................................................................................................................................ 126
5.6 Discussion ................................................................................................................................. 127

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE DIRECTIONS ....131

6.1 Conclusions ................................................................................................................................ 137

REFERENCES ................................................................................................................................. 139

APPENDIX A: PRELIMINARY CELL CULTURE EXPERIMENTS .......... 160
   A.1. Materials and methods ........................................................................................................ 160
      A.1.1. Bulls ............................................................................................................................ 160
      A.1.2. Cell isolation ................................................................................................................ 160
      A.1.3. Cell culture and hormone treatments ......................................................................... 160
      A.1.4. Statistical analyses ...................................................................................................... 161
   A.2. Results .................................................................................................................................. 161
   A.3. Discussion ............................................................................................................................ 162

APPENDIX B: COPYRIGHT PERMISSIONS ........................................... 164
   B.1. Co-Author release letter ....................................................................................................... 164
   B.2. Journal of Dairy Science release ....................................................................................... 166
List of Tables

Table 2-1. Primer sequences used to characterize genes in testicular tissues............................. 39

Table 2-2. Mean (± SEM) for various reproductive end points in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. .................. 51

Table 3-1. Diet costs (CAD$) for Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age................................................................. 67

Table 3-2. Costs of feed ingredients (December 2015) used for calculation of total diet costs.. 68

Table 3-3. Estimates of total sperm produced per day, number of resulting AI doses and the value of those doses from Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age................................. 77

Table 3-4. Mean (± SEM) ages (d) for various reproductive end points and progressive motility (%) at first freezable semen samples in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. 79

Table 3-5. Mean (± SEM) end points for in vitro fertilization using sperm from Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age. 80

Table 3-6. Mean (± SEM) viability results from Live/Dead sperm kit using flow cytometry in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 3 bulls/diet; three ejaculates per bull) from 2 to 31 wk of age................................................................. 81

Table 4-1. Composition of low-, and high-nutrition diets (as-fed; %) for Holstein bull calves from 8 to 32 wk of age................................................................. 92

Table 4-2. Primer sequences used to characterize gene expression in testicular tissues. .......... 94
List of Figures

Figure 1-1. Endocrine changes during reproductive development of the bull (based on Evans et al. 1995; Chandolia et al. 1997c; Rawlings et al. 2008)................................................................. 5

Figure 1-2. Leydig cell differentiation (adapted from Mendis-Handagama and Ariyaratne 2001). ........................................................................................................................................ 8

Figure 1-3. Testicular development during reproductive development of the bull calf (based on Evans et al. 1995; Chandolia et al. 1997c; Rawlings et al. 2008)........................... 11

Figure 1-4. Reproductive development of the bull calf (based on Evans et al. 1995; Chandolia et al. 1997c; Rawlings et al. 2008)................................................................. 13

Figure 1-5. Insulin/IGF-I signaling (adapted from Griffeth et al. 2014)........................................ 19

Figure 1-6. Summary of effects of IGF-I on the male reproductive axis (adapted from Zulu et al. 2002). ............................................................................................................... 23

Figure 2-1. Mean (± SEM) for BW, SC, and PTV in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. ........................................... 42

Figure 2-2. Mean (± SEM) PTV as a percentage of total BW, testicular pixel intensity and testicular vascular cone diameter in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. .................................................. 44

Figure 2-3. Mean (± SEM) total LH secretion, number of LH pulses and pulse amplitude of LH pulses during 10-h intensive blood sampling (every 15 min) in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age. ............... 45

Figure 2-4. Mean (± SEM) serum LH concentrations and total LH during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age................................................................. 46

Figure 2-5. Mean (± SEM) FSH secretion during 10-h intensive blood sampling (every 15 min) and FSH secretion during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age. .................................................... 48

Figure 2-6. Mean (± SEM) serum testosterone concentrations during 10-h intensive blood sampling (every 15 min) and during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age. .............. 49

Figure 2-7. Mean (± SEM) serum concentrations of IGF-I, insulin and leptin during 10-h intensive blood sampling (every 15 min) in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age....................................................... 50
Figure 2-8. Representative image of a round seminiferous tubule used to measure seminiferous tubule diameter in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. ................................................................. 52

Figure 2-9. Mean (± SEM) expression patterns (1/ΔC_q) of A) WT1, B) AR C) GATA4, and D) P27 in testes of adult Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. ............................................................................. 53

Figure 2-10. Mean (± SEM) expression patterns (1/ΔC_q) of A) 3β-HSD, B) IGF-I and C) IGF-IR in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age. ............................................................................. 54

Figure 3-1. Example flow cytometry output for sperm stained with SYBR14 and PI to evaluate viability. .................................................................................................................. 74

Figure 3-2. Percentage of morphologically normal sperm over time in Holstein bulls fed low-, medium-, or high-nutrition diets (top, middle and bottom panels respectively) from 2 to 31 wk of age. ............................................................................................................... 78

Figure 3-3. Representative 2D gel of sperm proteins from Holstein bulls fed low-, medium-, or high-nutrition diets (n = 3 bulls/diet) from 2 to 31 wk of age. ................................................................. 82

Figure 3-4. Representative 2D-gel of sperm proteins from Holstein bulls fed low-, medium-, or high-nutrition diets (left to right, n = 3 per group) from 2 to 31 wk of age. ......................................................... 83

Figure 4-1. Mean (± SEM) BW of Holstein bulls fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ................................................................. 96

Figure 4-2. Mean (± SEM) testes weight of Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ......................................................... 97

Figure 4-3. Representative images of tubular diameter of the seminiferous tubules of Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk and castrated at four ages. ................................................................. 98

Figure 4-4. Mean (± SEM) tubular diameter of Holstein bulls fed low- or high-nutrition diet (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ................................................................. 99

Figure 4-5. Mean (± SEM) expression (1/ΔC_q) of A) AR and B) P27 in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ............................................................................. 101

Figure 4-6. Mean (± SEM) expression (1/ΔC_q) of A) AMH, B) P450 and C) NCAM in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ............................................................................. 102
Figure 4-7. Mean (± SEM) expression (1/ΔCq) of A) PCNA and B) Ki67 in Holstein bulls fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages. ................................................................. 103

Figure 4-8. Representative images of IHC of seminiferous tubules of 24 wk-old calves immunolabelled for PCNA. .................................................................................................................. 104

Figure 4-9. Representative negative image of IHC of seminiferous tubules of 24 wk-old calves immunolabelled for PCNA and counter stained with DAPI. ....................................................... 104

Figure 4-10. Mean (± SEM) expression (1/ΔCq) of A) IGF-I and B) IGF-IR in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk and castrated at four ages. .............................................................................. 105

Figure 5-1. Bovine testes during Sertoli cell isolation procedure .............................................. 116

Figure 5-2. Bovine seminiferous tubules (40X) after enzyme digestion 2. ................................. 117

Figure 5-3. Bovine seminiferous tubules (40X and 100X) after enzyme digestion 3. ................. 118

Figure 5-4. Cells after passage through cell strainer (200X). ...................................................... 118

Figure 5-5. Phase contrast images of Sertoli cells in culture ...................................................... 123

Figure 5-6. Representative immunocytochemistry images of Sertoli cells stained for GATA4 and DAPI. ........................................................................................................................... 124

Figure 5-7. Western blot of GATA4 (45kDA) from bovine Sertoli cells ..................................... 125

Figure 5-8. Representative immunocytochemistry images of Sertoli cells immunostained for 3β-HSD and DAPI. ........................................................................................................... 125

Figure 5-9: Mean (± SEM) Sertoli cell number after 24 h of hormone treatment ......................... 126

Figure 5-10: Mean (± SEM) percentage of Sertoli cells expressing proliferation marker after 24 h of hormone treatment ........................................................................................................... 127
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3beta-Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-mullerian hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>AV</td>
<td>Artificial vagina</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CASA</td>
<td>Computer assisted sperm analysis</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus oocyte complex</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>Cq</td>
<td>Mean threshold cycle</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecoc’s modified eagle medium</td>
</tr>
<tr>
<td>DSP</td>
<td>Daily sperm production</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ESR</td>
<td>Epididymal sperm reserves</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescent isothiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor- I</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Insulin-like growth factor- II</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor- I receptor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding proteins</td>
</tr>
<tr>
<td>INSR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IVC</td>
<td><em>In vitro</em> culture</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IVM</td>
<td><em>In vitro</em> maturation</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology and Information</td>
</tr>
<tr>
<td>NHPP</td>
<td>National Hormone and Peptide Program</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinosite 3-kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A/ cAMP-dependent kinase</td>
</tr>
<tr>
<td>PTV</td>
<td>Paired testes volume</td>
</tr>
<tr>
<td>RC DC</td>
<td>Reagent compatible detergent compatible</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Scrotal circumference</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology domain 2</td>
</tr>
<tr>
<td>SOF</td>
<td>Synthetic oviduct fluid</td>
</tr>
<tr>
<td>TALPH</td>
<td>Tyrode albumin lactate pyruvate hepes buffer</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributylphosphine</td>
</tr>
<tr>
<td>TDN</td>
<td>Total digestable nutrients</td>
</tr>
<tr>
<td>TPI</td>
<td>Testicular pixel intensity</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor-1</td>
</tr>
</tbody>
</table>
Chapter One: **Introduction**

The United Nations projects that world population will increase by another 2.5 billion persons by 2050 to surpass 9 billion. Along with this substantial increase in population, there will be a corresponding increase in global demand for food, including an increase in demand for animal products (Speedy 2003). Proteins, including those from animals, are an integral part of a healthy balanced diet. Projected increases in protein consumption and global demand necessitate an increase in animal production. Reproduction has a key role in increasing quantity and efficiency of global food production. For example, it has been suggested that the most important factor increasing farm animal productivity since the 1940’s was adoption of breeding technologies (Johnson and Ruttan 1997). Increasing global and local demands are compelling the cattle industry to improve reproductive efficiency through research and development, providing substantial economic and social benefits.

### 1.1 Canadian artificial insemination (AI) industry

The Canadian dairy industry is a multi-billion dollar industry, internationally known for its superior genetics as well as reliable genetic evaluation and dairy cattle improvement programs (Government of Canada 2015). In 2008, 41% of the global export market for live purebred dairy cattle breeding stock and embryos originated from Canada (Government of Canada 2015) and in 2014, $158 million of dairy genetics (semen, embryos or live cattle) were exported from the country (Government of Canada 2015). The dairy industry is the third largest agriculture sector in Canada, after grains/oil seeds and red meats. In 2009, dairy production generated $13.6 billion in revenue, accounting for 15% of the Canadian food and beverage sector (Government of Canada 2015).
Holstein is the most common dairy breed in Canada, comprising 94% of dairy cattle (Government of Canada 2015); consequently, they comprise the majority of bulls in Canadian AI centers. Most AI sires are born and initially raised (until ~8 mo of age) on commercial dairy farms. Historically, once potential AI sires were brought to an AI center, they were trained for semen collection (via artificial vagina; AV) and the semen used to generate daughters, with resulting milk yield and phenotypic data collected to determine the sire’s genetic merit (progeny testing). This approach requires birth of two generations of offspring and therefore takes several years before the true genetic merit of a dairy bull can be identified. Consequently, bulls that reach puberty faster and/or maximize the number of insemination doses produced are of greater value to the AI industry.

Genomic information for selection of animals for breeding purposes has become increasingly used in the Canadian dairy industry. More than 60 traits are currently used to estimate a bull’s genetic merit from genomic evaluations of DNA profiles (Government of Canada 2015). Prediction of genetically superior bulls prior to birth through selective breeding has many advantages for the dairy industry. Furthermore, using genomics rather than progeny testing reduces costs and generation intervals and hastens genetic improvement by increasing accuracy of selection (Schaeffer 2006).

Genetic selection of young bulls creates increased economic pressure for these bulls to produce marketable semen doses at an earlier age. However, genetics are not the only consideration. There are indications that early-life management of bulls may influence their future reproductive potential. Unfortunately, many potential future AI sires arrive at the AI centre at body weights and body condition below optimal levels, since not all are well managed during the first few months of their lives. Presumably, a combination of genomic selection and
proper management could facilitate production of marketable semen at younger ages and more insemination doses, with positive economic effects for the AI industry.

1.2 Reproductive development of bulls

1.2.1 Endocrine changes during reproductive development of the bull

Post-natal reproductive development of the bull is divided into three periods: infantile, prepubertal, and pubertal. The infantile period occurs from 0 to 8 wk of age and is characterized by low secretions of both gonadotropins and testosterone. The prepubertal period (8 to 20 wk) is characterized by a transient increase in gonadotropin secretion (early gonadotropin rise) and a concurrent increase in testosterone secretion (Barth et al. 2008). Concentrations of Luteinizing hormone (LH) increase from 4 to 5 wk, peak at 12 to 16 wk, and then decline, reaching a baseline at 25 wk (Amann 1983; Evans et al. 1995). This early rise in LH has been attributed to increased LH pulse frequency (Evans et al. 1995; Bagu et al. 2004). At 4 wk of age, bull calves have, on average, less than one LH pulse per day, increasing to ~12 to 16 per day between 12 to 16 wk and decreasing to ~ 8 pulses per day by 28 wk (Amann and Walker 1983). Concentrations of LH during the prepubertal period affect sexual development and are inversely related to age at puberty (Amann 1983; Evans et al. 1995). The early gonadotropin rise may have a pivotal role in sexual development and maturation of the bull calf, as it occurs just prior to rapid testicular growth (Evans et al. 1995; Rawlings et al. 2008) and the magnitude of these gonadotropin secretions may be critical in initiation and magnitude of testicular development in bulls (Barth et al. 2008). Peripheral follicle stimulating hormone (FSH) concentrations generally increase during the early gonadotropin rise, and subsequently decrease to baseline by ~25 wk (Amann 1983; Evans et al. 1995). However, FSH secretion patterns seem more variable than LH
(Aravindakshan et al. 2000; Bagu et al. 2004), with some studies concluding that there is no consistent pattern of FSH secretion during the prepubertal period (McCarthy et al. 1979a; Amann and Walker 1983; Rawlings et al. 2008). Testosterone concentrations increase from 4 to 25 wk of age, ultimately suppressing gonadotropin concentrations due to negative feedback (Senger 2003; Rawlings et al. 2008).

The pubertal period extends from ~20 to 24 wk until puberty and is characterized by accelerated reproductive development, decreased gonadotropin secretion and increasing testosterone secretion (Lacroix and Pelletier 1979; McCarthy et al. 1979a; McCarthy et al. 1979b; Amann 1983; Amann and Walker 1983; Amann et al. 1986; Evans et al. 1996). Endocrine changes during reproductive development of bull calves are shown in Figure 1-1. In the post-pubertal bull, every gonadotropin-releasing hormone (GnRH) pulse is associated with pulsatile secretion of gonadotropins and testosterone, with approximately 4 to 8 pulses every 24 h (Senger 2003).
1.2.2 Testicular changes during reproductive development of the bull

The testicular parenchyma is divided into two compartments: the tubular compartment and the interstitial compartment (Senger 2003). The tubular compartment is comprised of the seminiferous epithelium, Sertoli cells, developing germ cells, peritubular cells and the basement membrane. The interstitial compartment is comprised of mesenchymal-like cells, fibroblasts, Leydig cells, peritubular cells, capillaries, lymphatic vessels, and connective tissue (Senger 2003).

In bull calves prior to 25 wk, testes are comprised of pre-spermatogonia, spermatogonia, Leydig cells, and undifferentiated Sertoli cells. At this stage of life, testicular growth occurs slowly. However, testicular development is most rapid between ~6 and 18 mo of age (Evans et al. 1995); therefore, it appears that there is a gonadotropin-independent mechanism regulating
testicular development (Brito et al. 2007c) as gonadotropin concentrations are relatively low during the prepubertal period. Histological changes include a marked increase in diameter and length of seminiferous tubules, proliferation and differentiation of germ cells, and development of adult Leydig cells (30 wk), Sertoli cells (30 to 40 wk), and mature sperm (32 to 40 wk) (Rawlings et al. 2008).

1.2.2.1 Leydig cell development

There are two distinct types of Leydig cells, fetal and adult, and there are currently two main hypotheses regarding how these populations are related (Griffeth et al. 2014). The first hypothesis is that a common progenitor population gives rise to both the fetal and adult Leydig cells, but adult cells remain dormant until pre-puberty (Barsoum et al. 2013; Svingen and Koopman 2013), whereas the second hypothesis is that independent cell lineages give rise to fetal and adult populations (Vergouwen et al. 1991; O'Shaughnessy et al. 2002).

There are five main cell types involved in the Leydig cell lineage: mesenchymal precursor cells, progenitor cells, newly formed adult Leydig cells, immature Leydig cells, and mature Leydig cells (Mendis-Handagama and Ariyaratne 2001; Chen et al. 2009). In bulls, both degenerating fetal Leydig cells and newly formed adult Leydig cells are present at birth; however, by 8 wk, only postnatal populations are present (Wrobel 1990). The first stage in Leydig cell differentiation is transformation of mesenchymal precursor cells to Leydig progenitor cells (Mendis-Handagama and Ariyaratne 2001). That the latter have the ability to produce androgens and express steroidogenic enzymes suggests they are committed to becoming Leydig cells. These spindle-shaped Leydig progenitor cells subsequently become round and are located towards the center of the interstitium (Mendis-Handagama and Ariyaratne 2001). These
cells are called newly formed adult Leydig cells and are distinguishable from further
differentiated immature Leydig cells and mature Leydig cells as they are smaller round cells.
Newly formed adult Leydig cells grow and acquire more cytoplasm and become immature
Leydig cells, which contain lipid droplets (Mendis-Handagama and Ariyaratne 2001). Adult
Leydig cells are responsible for production of testosterone. These cells subsequently become
mature Leydig cells and in bulls, this Leydig cell population is formed by 30 wk of age (Wrobel
1990). Stages of Leydig cell development are shown in Figure 1-2.

Many factors influence Leydig cell differentiation including LH, IGF-I, AR,
steroidogenic factor 1 (SF-1), and platelet-derived growth factor (PDGF). For proper
development of both types of Leydig cells, the nuclear receptor SF-1 is required (Karpova et al.
2015). AR is known to have an important role in the development of the adult Leydig cell
population. Recently a gain-of-function Sertoli cell AR mouse model has been used to
demonstrate that Sertoli cell AR regulates Leydig cell function and dictates Leydig cell numbers
(Hazra et al. 2013). Additionally, studies on AR knockout mice showed that AR is critical for
attaining normal adult Leydig cell population during testes development (Kilsoyne et al. 2014;
O’Shaughnessy et al. 2002). Additionally, PDGF is essential for the differentiation of the adult
Leydig cells as various PDGF gene studies have shown that without PDGF, the adult Leydig cell
population is severely affected (Basciani et al. 2010).
1.2.2.2 Sertoli cell development

Sertoli cells proliferate at two distinct intervals during maturation: during fetal or neonatal life and during the peripubertal period. There are two discrete Sertoli cell types, referred to as fetal and adult, which differ in morphology, function, and age at which they appear in the testis. In bulls, the fetal population of Sertoli cells is present at birth and starts undergoing accelerated cell division from 4 to 8 wk of age. This cell division subsequently declines and by 12 to 24 wk (depending on breed and maturation status), Sertoli cell proliferation stops, entering into the G0-phase for the remainder of the bull’s life (Abdel-Raouf 1960; Wrobel 2000). Undifferentiated Sertoli cells then begin to undergo terminal differentiation and maturation to form adult cells. These changes are characterized by morphological and functional changes, loss of proliferative ability, and formation of inter-Sertoli cell tight junctions (Tarulli et al. 2012). Main morphological changes include enlargement of the nucleus with a prominent nucleolus (Sharpe et al. 2003). The major hormone that regulates Sertoli cell development is FSH; it
influences Sertoli cell proliferation via cAMP-dependent kinase (PKA) and mitogen-activated protein kinase (MAPK) pathways (Crepieux et al. 2001). Bulls given exogenous FSH from 4 to 8 wk of age had increased testes size, likely due to greater Sertoli cell proliferation (Bagu et al. 2004). Since only immature Sertoli cells can proliferate, post-pubertal Sertoli cell number is determined before puberty. Efficiency of spermatogenesis is influenced by Sertoli cell number, therefore factors affecting Sertoli cell proliferation during the prepubertal period influence testis size and daily sperm production (Sharpe et al. 2003).

Many factors influence Sertoli cell number, including FSH, insulin-like growth factor-I (IGF-I), thyroid hormone, fibroblast growth factor, epidermal growth factor, transforming growth factor alpha, and glial cell line-derived neurotropic factor (GDNF; Hu et al. 1999; Griffeth et al. 2014). FSH is known to be one of the main factors affecting Sertoli cell proliferation during testes development (Orth 1984). It has been shown that transforming growth factor alpha, fibroblast growth factor and epidermal growth factor can stimulate Sertoli cell proliferation in vitro (Petersen et al. 2001; Jaillard et al. 1987). GDNF, together with FSH, also stimulates Sertoli cell proliferation in cultured rat testicular tissue (Hu et al. 1999). Thyroid hormone acts in the opposite direction of many of the previously mentioned factors, acting to suppress Sertoli cell proliferation and inducing Sertoli cell maturation (Holsberger and Cooke 2005). Hypothyroid neonatal rats have extended Sertoli cell proliferation and delayed maturation, leading to an increased Sertoli cell number in the adult animal (Holsberger and Cooke 2005). This can be contrasted to hyperthyroid neonatal rats, which have a shortened proliferation phase and therefore earlier Sertoli cell maturation.
1.2.2.3 Germ cell development

In prenatal bull calves, there is a high proportion of proliferating germ cells from days 50 to 80 of pregnancy, which are transitioning from primordial germ cells to prespermatogonia (Wrobel 2000). Thereafter, germ cells enter into a period of quiescence and no cell division occurs. During post-natal life, germ cell proliferation resumes by 4 wk of age and seminiferous tubular diameter increases from 40 to 80 µm from 4 to 15 wk (Wrobel 2000). GDNF, produced by Sertoli cells, is known to promote the self-renewal of the spermatogonia (de Rooij 2001). In accordance with increasing LH concentrations, there is increased testosterone production from the growing Leydig cell population, promoting testes growth and spermatogenesis. In Holstein bulls, gonocytes are the predominant germ cell at 12 wk, but by 20 wk, they are replaced by prespermatogonia and a-spermatogonia (Curtis and Amann 1981). Spermatocytes appear by 16 to 24 wk, with elongated spermatids present by 32 wk. Retinoic acid, cyclin D2, deleted in azoospermia-like (Dazl) and stem cell factor -c-kit, -have all been shown to play a role in the differentiation of prespermatogonia into A1 spermatogonia (de Rooij 2001). Final mature spermatozoa are first observed between 32 to 40 wk, signifying completed spermatogenesis (Curtis and Amann 1981). The adult male is able to continuously produce spermatozoa through spermatogenesis, a process made possible by the stem cell niche. The niche of the spermatogonia stem cells provides a micro environment that controls and maintains the self-renewing properties of the stem cells (Spradling et al. 2001).

Testicular weight increases rapidly from 5 to 12 mo; the rate of increase begins to decrease at 16 mo, with 90% of final testis weight by 24 mo (Barth and Ominski 2000; Brito 2006). Testicular development during reproductive development of a bull calf is shown in Figure 1-3.
1.2.3 Puberty

In a bull, puberty generally refers to the ability to produce spermatozoa capable of fertilizing an oocyte. Furthermore, the bull must be able to exhibit sexual behaviours required to breed a female, including having adequate “penile development” to achieve penetration and ejaculation (Lunstra et al. 1978; Amann 1983). Another common definition of puberty for bulls is the age at which the first ejaculate containing a minimum of 50 million sperm with > 10% motility is collected (Wolf et al. 1965). On average, bulls reach puberty when their scrotal circumference (SC) is 28 cm; therefore, this age is often used as a proxy for puberty, as measuring SC is easier than collecting semen (Lunstra et al. 1978; Rawlings et al. 2008).
Puberty in bulls is regulated by an interaction of the hypothalamus, pituitary (hypophysis) and testis (hypothalamo-hypophyseal-testis axis). The hypothalamus secretes GnRH in a pulsatile manner, which induces a pulsatile release of LH and FSH from gonadotrophs in the anterior pituitary. Gonadotropins enter systemic circulation and reach the testes where they exert their effects. Under the influence of LH, Leydig cells in the testes release testosterone in a pulsatile manner (testosterone is subsequently converted to dihydrotestosterone and estradiol in Sertoli cells). Testosterone regulates Sertoli cell function by non-classical steroid signaling (Walker 2010). High testosterone concentrations in the seminiferous tubule are required for spermatogenesis, and FSH is mainly involved in regulation of Sertoli cell function (Walker and Cheng 2005). Testosterone, in combination with estradiol, down-regulates GnRH release via negative feedback from testis. In prepubertal bulls, the hypothalamus is highly sensitive to the negative feedback effects of testosterone and estradiol. Ultimately, puberty occurs due to decreased sensitivity of the hypothalamus to negative feedback of testosterone and estrogen, with concomitant increases in concentrations of GnRH, LH, FSH, and testosterone (Amann 1983). All aspects of reproductive development of the bull calf are now summarized in one comprehensive figure, combining all information presented thus far (Figure 1-4).
1.2.4 Hastening puberty through endocrine manipulation

Endocrine manipulation can modulate age at puberty in bulls, providing insights into mechanisms controlling reproductive development and puberty in bull calves. Early-maturing bulls have higher LH concentrations during the early gonadotropin rise when compared to late-maturing bulls (Evans et al. 1995). Furthermore, suppressing the early rise of LH (by giving a GnRH agonist) delays testicular development, reinforcing the critical role of the early LH rise on testicular development (Chandolia et al. 1997a). Furthermore, exogenous GnRH has also been given to try to hasten puberty. Many early attempts were not successful, as the GnRH may have been given too close to puberty (Amann 1983). However, in subsequent studies, giving bulls GnRH every 2 h from 4 to 6 wk of age hastened onset of sexual maturity and increased testes
size (Chandolia et al. 1997b). Furthermore, giving GnRH twice a day from 4 to 8 wk produced similar results, with bulls that were younger at puberty (Madgwick et al. 2008). Exogenous FSH from 4 to 8 wk also had a similar effect as GnRH and increased number of Sertoli cells (Bagu et al. 2004). Combining all of these endocrine studies, it is clear that the early gonadotropin rise has a critical role in sexual maturity in bulls.

1.3 Links between nutrition and reproduction

In the 1940’s, Dr. Barker identified that maternal nutrition affected postnatal growth and development leading to his developmental origins of health and disease hypothesis, which has since been well established (Barker and Osmond 1986; Boo and Harding 2006). The Barker’s hypothesis originated from observations of poor maternal nutrition and low infant mortality in Europe during the Second World War and high coronary heart disease decades later. Poor prenatal nutrition, as a result of inadequate maternal nutrition during gestation, reduces reproductive development and fertility (Rae et al. 2002). In male sheep and rats, inadequate nutrition during gestation delayed sexual maturity, decreased testicular volume, and decreased plasma testosterone concentrations (Da Silva et al. 2001; Zambrano et al. 2005).

Additionally there are well-established links between nutrition and reproduction specifically (Dupont et al. 2014). There is experimental evidence that GnRH neurons communicate with a neural system called the metabolic sensor, which relays nutritional status of the body to GnRH neurons by detecting metabolic hormones (e.g. leptin, IGF-I, insulin, and growth hormone) and nutrients (Blache et al. 2000; Brito et al. 2007a; Brito et al. 2007c; Brito et al. 2007b). This communication enables GnRH neurons to overcome negative feedback effects of testosterone and estradiol, and initiate pulsatile release of GnRH. Research on effects of
nutrition on reproduction and development is usually organized into three general time frames: prenatal, prepubertal (early-life) and post-pubertal.

It is well established that overfeeding post-pubertal bulls can reduce reproductive performance (Young 1974; Brown 1994). This was attributed to increased fat in the scrotal stem, which presumably interfered with testicular thermoregulation, thereby reducing semen quality (Brown 1994). Feeding Herford and Angus bulls high-energy diets from 6 to 24 mo of age reduced their fertility (Coulter and Kozub 1984). In humans, adult male obesity has been linked to decreased fertility (Hammoud et al. 2008; Du Plessis et al. 2010). However, in a recent study, body mass index in humans was not associated with sperm morphology or motility; it is noteworthy that only extreme obesity suppressed male fertility (Chavarro et al. 2010).

Overall, the most profound effects of nutritional modulation on reproductive potential of bulls are during early-life (prepubertal period). Enhanced prepubertal nutritional modulation (energy and protein) promoted reproductive development. Beef bull calves fed a high level of nutrition from 10 to 30 wk of age have increased testicular weight and sperm production by 74 wk of age compared to control bull calves fed only a recommended level of nutrition (Brito et al. 2007b). Beneficial effects of early-life nutrition on post-pubertal bulls were attributed to increased LH secretion by 22 wk of age (Brito et al. 2007a). Furthermore, since detrimental effects of restricted feeding from 10 to 26 wk were not overcome by nutritional supplementation during the peri-pubertal period, it was concluded that early-life nutrition predetermines age at puberty and testicular size at sexual maturity (Brito et al. 2007a).

In dairy bulls fed various levels of energy from early-life to 4 y of age, the low-nutrition group temporarily (~80 wk) had lower average sperm motility than other groups. However, this difference subsequently disappeared with increasing age (Flipse and Almquist 1961).
Furthermore, in underfed 3-y-old Holstein bulls, the number of spermatozoa collected was 77% of that from normally fed controls (VanDemark et al. 1964).

Effects of early-life nutrition on reproduction are consistent across species and sexes. In rams, level of nutrition affects gonadotropin secretion (Blache et al. 2000) and in heifers, increasing energy intake during the prepubertal period decreased age at puberty (Gasser et al. 2006). In a recent study, feeding Holstein bulls high-energy diets from 8 to 32 wk advanced some aspects of sexual maturation (LH pulses and SC), although age at puberty and sperm production were not affected (Harstine et al. 2015). It is noteworthy that most of the previous research in this field was done in beef cattle. It is therefore important to extend this knowledge to the dairy industry and determine effects of early-life nutrition on future reproductive potential of dairy bulls.

1.3.1 Insulin-like growth factor-I (IGF-I)

Among metabolic hormones, IGF-I has received considerable attention due to its effects on the hypothalamo-hypophyseal-gonadal axis and reproduction. IGF-I is a member of the insulin-like family of growth factors, which also includes insulin and Insulin-like growth factor-II (IGF-II; Griffeth et al. 2014). It has important autocrine and paracrine roles at the cellular level by regulating mitosis, apoptosis, and cellular differentiation (Butler et al. 1998; Griffeth et al. 2014).

IGF-I concentration has been suggested to predict both nutritional status and reproductive status in dairy cattle (Zulu et al. 2002). In females, IGF-I concentrations during the early postpartum period (characterized by negative energy balance, NEB) correlate with energy status and are therefore low. Holstein cows fed lower levels of energy had lower plasma IGF-I (Lucy et
al. 1992). In beef bulls, low-nutrition diets reduced circulating IGF-I concentrations and delayed the rise of testosterone (there is a temporal association between concentrations of LH and IGF-I; Barth *et al*. 2008), highlighting the role of the latter hormone in regulation of male reproduction. Furthermore, calves on restricted daily gain diets prior to puberty had lower serum IGF-I concentrations (Renaville *et al*. 2000). This leads to further questions regarding the role of IGF-I in reproduction, specifically testicular development.

Although IGF-I is synthesized by almost all mammalian tissues, it is primarily produced in the liver (Griffeth *et al*. 2014). Expression of IGF-I is regulated by growth hormone (GH) in many tissues, although it also has GH-independent functions and its expression is also regulated by factors other than GH (Le Roith *et al*. 2001). In circulation, IGF-I is bound to one of six IGF binding proteins (IGFBP), which affect IGF’s ability to bind to its receptors, tissue delivery, and maintenance in circulation (Butler *et al*. 1998; Forbes *et al*. 1998). The action of IGF-I is mediated primarily via a type I IGF-I receptor (IGF-IR), a member of the insulin receptor family of receptor tyrosine kinases (Adams *et al*. 2000; Griffeth *et al*. 2014). Similar to other members of this receptor’s family, the IGF-IR has a heteromeric \( \alpha_2\beta_2 \) structure, with the ligand-binding domain on the extracellular \( \alpha \) subunit. The \( \beta \) subunit is largely intracellular, containing the tyrosine kinase domain (Butler *et al*. 1998).

### 1.3.1.1 IGF-I signaling

Several pathways and processes are activated following activation of receptors in the insulin/IGF family (Butler *et al*. 1998; Griffeth *et al*. 2014). Activation of the IGF-IR activates its tyrosine kinase domain, which causes phosphorylation of the \( \beta \) subunit, allowing multiple adaptor proteins to bind to the cytoplasmic region of the IGF-IR, including members of the
insulin receptor substrate (IRS) family, Src homology 2 domain containing transforming protein 1 (SHC), and the p85 subunit of phosphoinositol-3’ kinase (PI3K) (Butler et al. 1998; Griffeth et al. 2014). The two major signaling pathways associated with IGF-I are PI3K and MAPK, both of which can be activated via adaptor proteins from the IRS family (Griffeth et al. 2014). Furthermore, both of these pathways are involved in cell proliferation, differentiation, metabolism and cell survival.

The PI3K pathway is an important mediator for the anti-apoptotic effects of IGF-I (Butler et al. 1998). The p85 subunit of PI3K binds to IRS proteins and activates the p110 subunit (catalytic subunit) of PI3K (Butler et al. 1998). Active PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2), converting it to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane, enabling PDK1 to phosphorylate protein kinase B (PKB, or better known as AKT; cellular homolog of murine thymoma virus akt8 oncogene). The now active AKT dissociates from PIP3 and phosphorylates a number of cytosolic and nuclear proteins, including mammalian target of rapamycin (mTOR), a key regulator of increased protein transcription and translation.

In addition, IGF-I activates the MAPK pathway, which seems to affect proliferation, differentiation and cell survival (Pitetti et al. 2013). Interaction of IGF-I with IGF-IR phosphorylates its Src homology domain 2 (SH2). Consequently, this domain acts as a docking site for the SH2 domain of the adaptor protein growth factor receptor-bound protein 2 (GRB2), which activates Ras via Ras’s GDP-GTP exchanger SOS (Butler et al. 1998). The activated Ras acts as the start of the signaling cascade leading to activation of the rest of the kinases in the MAPk pathway (RAF/MEK/ERK). The two major signaling pathways for IGF-I are shown in Figure 1-5.
Recent studies have detected another level of complexity in the insulin/IGF-I signaling pathways, suggesting that both the insulin receptor and IGF-IR also have nuclear (genomic) roles (Sarfstein and Werner 2013). They are able to function similar to transcription factors as they can translocate to the nucleus. Although this research is in its infancy, it could have widespread implications for further understanding of many cellular events in which these receptors are involved.
1.3.1.2 IGF-I and reproduction

It is well known that IGF-I has critical roles throughout reproductive development and into adulthood, starting as early as the sex-determining stage of the developing embryo. Male mice mutant for insulin receptor (INSR) and IGF-IR develop ovaries and a completely feminized phenotype, suggesting that these receptors are required for male sex determination (Nef et al. 2003; Pitetti et al. 2013). It was reported that “IGF-I null males are infertile dwarfs and exhibit a reduction of greater than 80% in both spermatogenesis and serum testosterone levels” (Baker et al. 1996). Additionally, IGF-I has roles in functioning of the central nervous system, particularly at the hypothalamus and pituitary levels (D'Ercole et al. 1996; Zulu et al. 2002). Receptors for IGF-I are present in GnRH neurons; the number of these receptors increases during sexual development (Daftary and Gore 2004). Furthermore, IGF-I can stimulate GnRH release by the hypothalamus and exogenous IGF-I stimulates LH release from the pituitary in sheep (Hiney et al. 1996; Zhen et al. 1997; Adam et al. 1998).

It is becoming increasingly apparent that cells within the testes produce IGF-I and that this local production has both autocrine and paracrine functions to regulate testicular function (Griffeth et al. 2014). In rodents, LH stimulates IGF-I synthesis in Leydig cells and IGF-I up-regulates secretion of testosterone from Leydig cells (Rouiller-Fabre et al. 1998). In addition, synthesis of IGF-I in both Leydig (Wang and Hardy 2004) and Sertoli cells (Cailleau et al. 1990) and the presence of both IGF-I and its receptors in Leydig cells, Sertoli cells and spermatocytes in various species (Hansson et al. 1989; Wang and Hardy 2004; Barth et al. 2008; Villalpando et al. 2008) are consistent with important autocrine and paracrine functions of this hormone in these cells.
1.3.1.3 IGF-I and Leydig cells

Both IGF-I and LH are critical factors in determining Leydig cell numbers and their steroidogenic capacity. Furthermore, LH appears to not be a direct mitogenic factor of Leydig cells; in contrast, it apparently acts via IGF-I to control proliferation of Leydig cells (Wang and Hardy 2004). This potential interaction between LH and IGF-I was suspected when IGF-I null mice had fewer and smaller Leydig cells and lower serum testosterone concentrations in adulthood (Wang and Hardy 2004). Proliferation and differentiation of Leydig cells from their precursor cells are dependent on IGF-I signaling; IGF-I appears to mediate effects of LH as LH alone is less effective in stimulating Leydig cell proliferation (Wang and Hardy 2004). It was noted that there was potential “cross-talk between the insulin and LH signaling pathways at the level of PI3K/AKT pathway in the rat ovary” (Carvalho et al. 2003). Furthermore, they noted that “simultaneous infusion of insulin and LH induced higher phosphorylation levels of JAK2, STAT5b, IRS-I and AKT compared to the individual effects of these hormones in the whole ovary of normal rats” (Carvalho et al. 2003). Although this synergistic effect of LH and IGF-I has not been reported in the testis, this provides a possible explanation regarding which signaling cascades are affected and how LH acts via IGF-I to control proliferation of Leydig cells. In addition, IGF-I has critical autocrine/paracrine roles in controlling adult Leydig cell numbers and functions by promoting both proliferation and differentiation of precursors in the Leydig cell lineage (Wang and Hardy 2004; Villalpando et al. 2008; Hu et al. 2010). Moreover, signaling through IGF-IR has an important role in Leydig cell survival; in that regard, IGF-I seems to decrease cell apoptosis at all stages of Leydig cell development (Colon et al. 2007).
1.3.1.4 IGF-I and Sertoli cells

In the mouse, IGF-I increases Sertoli cell proliferation during embryonic days 14 to 18 (Villalpando et al. 2008). In culture, mouse Sertoli cells with a conditional knockout for IGF-IR had increased death and decreased proliferation, which decreases the number of viable Sertoli cells and highlighted the importance of IGF-I in regulation of Sertoli cell function (Froment et al. 2007). Sertoli cell-specific knockouts of IGF-IR and INSR resulted in mice with 75% smaller testes, attributed to reduced proliferation of immature Sertoli cells prior to reproductive maturation (Pitetti et al. 2013). Although FSH is one of the major hormones that regulates Sertoli cell proliferation, there is new evidence that the IGF-I pathway may be critical for mediating FSH’s action. In immature Sertoli cells, insulin/IGF signaling pathways are required for FSH to mediate its proliferative effects (Froment et al. 2007; Pitetti et al. 2013). Furthermore, FSH action is known to occur via a G protein-coupled receptor; however, its actions appear to be interconnected with the IGF pathway. This is potentially occurring through common downstream signaling pathways (Griffeth et al. 2014). In Sertoli cells, FSH amplifies IGF-I mediated PI3K/AKT signaling (Khan et al. 2002; Pitetti et al. 2013). Furthermore, AKT, in the PI3K pathway, has been suggested as the step where FSH and IGF-I mediated effects interact and it was reported that the “INSR and the IGF-IR are required for AKT expression and activation in Sertoli cells” (Pitetti et al. 2013). In granulosa cells, IGF-IR activity is required for FSH-induced phosphorylation of AKT (Pitetti et al. 2013; Zhou et al. 2013). This data provides evidence that FSH interacts with IGF signaling pathways to enhance stimulation of the PI3K/AKT pathway and secretion of IGF-I by Sertoli cells. Pitetti et al. suggested that this information supported the belief that local production of insulin/IGF-I in the testis “is the major intratesticular signal regulating Sertoli cell number, testis size and sperm output in mammals” (Pitetti et al. 2013).
Other researchers have implicated MAPK pathway as another potential way by which IGF-I promotes proliferation of Sertoli and Leydig cells of embryonic testes (Villalpando et al. 2008).

Overall effects of IGF-I on the reproductive axis are summarized in Figure 1-6.

Figure 1-6. Summary of effects of IGF-I on the male reproductive axis (adapted from Zulu et al. 2002).

Although effects of nutrition on several aspects of reproduction have been documented, several unanswered questions remain. In order to address some of these questions and provide some insight for the Canadian AI industry, this thesis explored the following aims.
1.4 Aims, hypotheses, objectives, and highlights

The overall aim of this thesis was to determine effects of early-life nutrition on reproductive potential of Holstein bulls.

**Aim 1:** To determine effects of early-life nutrition on serum concentrations of reproductive hormones, testicular size, age at puberty, and testicular characteristics in Holstein bulls.

**Specific hypotheses:**

1. A high-nutrition diet during early-life enhances secretion of reproductive hormones in Holstein bull calves.
2. Holstein bull calves on a high-nutrition diet will be younger at puberty and will have larger testes, and greater sperm production, than those on a low-nutrition diet.
3. Holstein bull calves maintained on a high-nutrition diet will have increased numbers of Sertoli cells, resulting in larger testes at puberty compared to bulls on a low-nutrition diet.
4. Holstein bull calves maintained on a high-nutrition diet will have higher expression of IGF-I and IGF-IR in their post-pubertal testes compared to bulls maintained on a low-nutrition diet.

**Specific objectives:**

1. To determine effects of early-life nutrition on serum profiles of reproductive and metabolic hormones prior to and following GnRH challenge during prepubertal reproductive development.
2. To determine effects of early-life nutrition on: testicular size, age at puberty, age at sexual maturity, daily sperm production, and epididymal sperm reserve.
3. To determine effects of early-life nutrition on expression of Sertoli cell markers in post-pubertal testes.

4. To evaluate effects of prepubertal nutrition on expression of IGF-I and IGF-IR in post-pubertal testes.

To achieve Aim 1, first I conducted a preliminary study to gain experience in experimental approaches and sample collection. Effects of early-life nutrition on reproductive development and post-pubertal testicular characteristics are described in Chapter 2.

**Highlights:**

- Bulls on high-nutrition diets had an early and more pronounced early gonadotropin rise.
- Bulls on a high-nutrition diet from 2 to 31 wk were younger at puberty and had larger testes than those fed a low-nutrition diet.
- IGF-I concentrations were significantly higher in high-nutrition bulls than the other two treatment groups, throughout the differential feeding period.
- Most of the data in this chapter has been published in *Journal of Dairy Science* (Dance et al. 2015).

**Aim 2:** To determine effects of early-life nutrition on semen characteristics of dairy bulls and estimate costs versus revenue associated with increased early-life feeding and resulting larger testes.
**Hypothesis:** A high-nutrition diet fed to Holstein bulls during early-life will not alter semen quality compared to bulls on a low-nutrition diet.

**Specific objectives:** To determine effects of early-life nutrition on:

- Age at maturity and age at first freezable semen.
- Post-thaw sperm characters (sperm viability, sperm protein expression, and *in vitro* fertilizing characteristics).
- Economic benefits.

Results of this study are provided in Chapter 3.

**Highlights:**

- There were no differences in semen quality among treatment groups.
- Age at maturity, age at first acceptable post-thaw, sperm post-thaw viability (live cells) and post-thaw fertilization ability were not significantly different among groups.
- Substantial increase in potential profit from high- versus low-nutrition bulls, even when taking into account additional feed costs.
- This study is in press in *Theriogenology*

**Aim 3:** To determine effects of early-life nutrition on reproductive development and prepubertal testicular characteristics.

**Specific hypotheses:**

1. Holstein bull calves maintained on a high-nutrition diet will have higher content of IGF-I and IGF-IR in their testicular tissues.
2. A high-nutrition diet will increase proliferation and differentiation of Sertoli cell precursors.

**Specific objectives:**

1. Evaluate testicular expression of IGF-I and IGF-IR in low- versus high-nutrition groups.
2. Evaluate proliferation and differentiation of Sertoli cell precursors.

Results of this study are provided in Chapter 4.

**Highlights:**

- Results were consistent with Chapter 3 in terms of testes size and body weight.
- Bulls fed a high-nutrition diet had greater expression of $AR$ and $P27$ (mature Sertoli cell markers) at 24 wk of age compared to those on the low-nutrition diet.
- Bulls on the high-nutrition diet had greater expression of $Ki67$ and $PCNA$ (cell proliferation markers) at 24 wk of age compared to those on a low-nutrition diet.
- IGF-I expression was higher in high-nutrition bulls at 24 wk.

**Aim 4:** To determine effects of IGF-I on Sertoli cells of prepubertal testis in culture (to understand potential mechanisms leading to larger testes in the high-nutrition group).

**Specific hypothesis:**

- IGF-I increases proliferation and differentiation of Sertoli progenitor cells in Holstein bull calves.
- IGF-I potentiates effects of FSH on Sertoli cells, enhancing Sertoli cell proliferation in Holstein bull calves.
Specific objectives:

1. Isolate Sertoli cells from Holstein bull calves
2. Investigate the effects of IGF-I and FSH on proliferation of Sertoli cells

Results of this study are provided in Chapter 5.

Highlights:

• Successfully isolated Sertoli cells from Holstein bull calves at 8, 16, 24, and 32 wk of age.
• IGF-I and FSH (in combination) increased Sertoli cell numbers in culture.
• IGF-I, alone or in combination with FSH, increased Sertoli cell proliferation.
• This study has been submitted to Reproduction, Fertility and Development.
Chapter Two: **Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls**

2.1 **Preamble**

The majority of the study described in this chapter was published in *Journal of Dairy Science* (J Dairy Sci 2015; 98:987-998). However, seminiferous tubule diameter and testicular gene expression experiments were not included in that publication and therefore have been added.

Title: Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls.

Alysha Dance, Jacob Thundathil, Randy Wilde, Patrick Blondin, and John Kastelic

A.D preformed 85% of experiments, analyzed 95% of the results and contributed 95% of the writing. J.T. supervised the project and reviewed the manuscript, R.W. supported all of the animal work and assisted with the animal portion of the experiments, P.B. supported the project and reviewed the manuscript and J.K. co-supervised the project and reviewed the manuscript. Hormone assays were performed at the University of Saskatchewan.

2.2 **Abstract**

Holstein bull calves often reach AI centers in suboptimal body condition. Early-life nutrition is reported to increase reproductive performance in beef bulls. The objective was to determine whether early-life nutrition in Holstein bulls had effects similar to those reported in beef bulls. Twenty-six Holstein bull calves were randomly allocated into three groups at
approximately 1 wk of age to receive a low-, medium-, or high-nutrition diet, based on levels of energy and protein, from 2 to 31 wk of age. Calves were on their respective diets until 31 wk of age, after which they were all fed a medium-nutrition diet. To evaluate secretion profiles and serum concentrations of hormones, a subset of the bulls were subjected to intensive blood sampling every 4 wk from 11 to 31 wk of age. Testes of all bulls were measured once a month; once SC reached 26 cm, semen collection was attempted (electroejaculation) every 2 wk to confirm puberty. Bulls were maintained until approximately 72 wk of age and then slaughtered at a local abattoir. Testes were recovered and weighed. Bulls fed the high-nutrition diet were younger at puberty (high = 324.3 d, low = 369.3 d; P < 0.05), and had larger testes for the entire experimental period than bulls fed the low-nutrition diet. Bulls fed the high-nutrition diet also had an earlier and more substantial early rise in LH than those fed the low-nutrition diet and had increased concentrations of IGF-I earlier than the bulls fed the low-nutrition diet. Furthermore, we detected a temporal association between increased IGF-I concentrations and an early LH rise in bulls fed the high-nutrition diet. Therefore, we inferred that IGF-I had a role in regulating the early gonadotropin rise (in particular, LH) and thus reproductive development of Holstein bulls. Overall, these results supported our hypothesis that Holstein bull calves fed a high-nutrition diet reach puberty earlier and have larger testes than those fed a low-nutrition diet, and they provided clear evidence that nutritional modulation of Holstein bull calves during early-life had profound effects on reproductive development.

**Key words:** Holstein bulls, nutrition, puberty, testes
2.3 Introduction

Most bulls in Canadian AI centers are Holstein. Future AI sires are typically born and raised on commercial dairy farms and moved to an AI center at approximately 8 mo of age. As soon as post-thaw semen quality is acceptable, semen is collected, cryopreserved, and used in AI programs for progeny testing. Bulls that reach puberty faster (facilitating progeny testing) or produce more doses of semen (greater profitability), or both, are clearly desirable. Regardless, many future potential AI sires arrive at the AI center in suboptimal body condition.

The effects of nutrition on the onset of puberty in beef bulls have been studied (Wolf et al. 1965; Brito et al. 2007a; Brito et al. 2007b; Brito et al. 2007c). Beef bull calves fed a high level of nutrition (both protein and energy) during early-life had increased (~20-25%) testicular weight and sperm production by 74 wk of age. Therefore, determining the effects of early-life nutrition on puberty and sperm production in Holstein bulls could be of great benefit to the AI industry.

Reproductive development of the bull can be divided into three periods: infantile, prepubertal and pubertal. The infantile period (0 to 8 wk of age) is characterized by low secretions of both gonadotropins and testosterone (Amann et al. 1986; Rawlings et al. 2008). Thereafter, during the prepubertal period (8 to 20 wk), there is a transient increase in blood gonadotropin concentrations (early gonadotropin rise) and a concurrent increase in testosterone secretion (Amann and Walker 1983; Barth et al. 2008; Rawlings et al. 2008). Peripheral LH concentrations start to increase at 4 to 5 wk, peak at 12 to 16 wk, and then decline, reaching a baseline at 25 wk (Amann and Walker 1983; Barth et al. 2008). It is noteworthy that LH concentrations during the prepubertal period affect sexual development and are inversely correlated with age at puberty (Amann and Walker 1983; Evans et al. 1995). Serum FSH
concentrations generally increase during the early rise (less marked than corresponding increases in LH), and decrease to baseline by approximately 25 wk (Amann and Walker 1983; Evans et al. 1995).

The early gonadotropin rise may play a pivotal role in sexual development and maturation of the bull calf (Rawlings et al. 2008). Specifically, the magnitude of prepubertal gonadotropin secretion may be critical in the initiation and extent of testicular development in bulls (Barth et al. 2008). Before 25 wk of age, testicular growth occurs slowly. Thereafter (pubertal period), there is rapid testicular development through puberty, despite low serum gonadotropin concentrations, suggesting that gonadotropin-independent mechanisms regulate testicular development in bulls. Ultimately, decreased sensitivity of the hypothalamus to testosterone and estrogen, with a concomitant increase in concentrations of GnRH, LH, FSH, and testosterone, culminate in puberty.

Experimental evidence indicates that GnRH neurons communicate with a neural system (so called metabolic sensor) that relays nutritional status to GnRH neurons by detecting concentrations of metabolic hormones (e.g. leptin, IGF-I, insulin, and growth hormone) and nutrients (Blache et al. 2000). Apparently, this communication enables GnRH neurons to overcome the negative feedback effects of testosterone and estradiol, and initiate pulsatile release of GnRH. Determining what metabolic hormones influence GnRH concentrations is important to elucidate the effects of nutritional status on reproduction. For example, concentrations of IGF-I are higher during the early gonadotropin rise in beef bulls (Brito et al. 2007a); indeed, because IGF-I concentrations are directly related to LH concentrations, it is thought that IGF-I may be involved in regulating the early gonadotropin rise in bulls (Brito et al. 2007c).
Most of the previous research on the effects of early-life nutrition on reproductive development in bulls was done in beef cattle. The overall objective of this study was to determine whether early-life nutrition in Holstein bulls had effects similar to those reported in beef bulls. We hypothesized that the Holstein bull calves on a high-nutrition diet will have greater LH concentrations during the prepubertal phase, reach puberty earlier, and have larger testes than those maintained on a low-nutrition diet.

2.4 Materials and methods

2.4.1 Bulls and treatments

Twenty-six Holstein bull calves were randomly allocated into three groups at approximately 1 wk of age to receive either a low-, medium-, or high-nutrition diet, from 2 to 31 wk of age. Typically calves raised in intensive systems are fed milk (twice daily) to 10% of the calves’ body weight (BW) (approximately 6 L of milk per day; Jasper and Weary 2002). Therefore, in the present study, calves were fed milk (4, 6 and 8 L/d in the low-, medium- and high-nutrition groups, respectively), from 2 to 8 wk of age and thereafter transitioned onto diets that were based on barley silage (the forage source for all diets). All diets contained 1.6% vitamin-mineral pre-mix (as-fed). The low-nutrition diet (n = 8) consisted of barley silage (plus pre-mix, but no concentrate) and had 12.2% crude protein (CP) and 62.9% total digestible nutrients (TDN; note that for this and all other diets, CP and TDN are reported on a dry matter (DM) basis). The medium-nutrition diet (n = 9) contained 4.8% rolled barley, 4.8% rolled corn, 3.8% canola meal and 3.8% soybean meal (overall, 17.0% CP and 66.0% TDN). The high-nutrition diet (n = 9) consisted of 9.7% rolled barley, 9.7% rolled corn, 7.6% canola meal and 7.6% soybean meal (20.0% CP and 67.9% TDN). These diets were formulated and classified as
low-, medium-, and high-nutrition levels, based on the results of a preliminary study, diets used in a previous study (Brito et al. 2007a; Brito et al. 2007c), and on National Research Council requirements (NRC; NRC 2001). The high-nutrition group was fed ad libitum and, based on their intake, the same amount of feed (on an as-fed basis) was offered to the low- and medium-nutrition groups. Calves were on their respective diets until 31 wk of age, after which they were all fed the medium-nutrition diet. This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the Lethbridge Research Centre Institutional Animal Care Committee.

2.4.2 Testicular characteristics

Once monthly, from 8 to 71 wk of age, all bulls were weighed and SC was determined with a Coulter Scrotal Tape (Trueman Manufacturing; Edmonton, AB, Canada). In addition, the length and width of each testis were measured with calipers and paired testes volume (PTV) was calculated using the following formula: $PTV = 0.5236 \times \text{length} \times \text{width}^2$ (Bailey et al. 1998). The PTV as a percentage of the overall BW was also calculated for each bull. Concurrent with determination of testicular size, testes were examined by conventional diagnostic ultrasonography, as described (Brito et al. 2012). Briefly, two images (testicular parenchyma and testicular vascular cone) from each testis of each bull were electronically captured and then recorded. For testes, the transducer was held vertically (parallel to the long axis of the testes) on the caudal surface of the scrotum and aligned so that the mediastinum was readily apparent before an image of each testis was captured. Testicular pixel intensity (TPI) was determined on a scale of 1 (white) to 255 (black); therefore, reduced TPI corresponded to increased tissue echodensity (brightness). The area for pixel analysis was selected by drawing a rectangle 0.5 to
1.0 cm deep into the parenchyma, where it appeared homogeneous; the area above the mediastinum was selected, and edges of the image were avoided (Gabor et al. 1998). For the testicular vascular cone, the transducer was held horizontally, and a cross-sectional image of each cone was recorded. The average diameter of the testicular vascular cones was also determined.

### 2.4.3 Blood samples and hormone analyses

To evaluate serum hormone concentrations and secretion profiles, a subset of the bulls (n = 6 per group) were subjected to intensive blood sampling every 4 wk (the same bulls were used for each sampling period) from 11 to 31 wk of age. Indwelling catheters were placed (into a jugular vein) one afternoon, and starting very early the following morning, blood was collected every 15 min for 11.5 h. At 10 h after the start of blood collection, GnRH (0.04 µg/kg IV; Fertagyl 2, Merck Animal Health, Intervet Canada Corp., Kirkland, QC, Canada) was given to stimulate the release of LH and testosterone; thereafter, blood sampling was continued for another 1.5 h. All blood samples were maintained at room temperature overnight to allow them to clot; the following morning, clots were removed and the tubes centrifuged (3480 × g for 20 min at 20 °C). Serum from every sample was removed with a pipette, placed separately in a storage tube, frozen, and stored until analyzed. Every sample was assayed to determine serum LH concentrations, whereas a pooled sample from the first 10 h for each bull was used to evaluate serum concentrations of FSH, testosterone, insulin, IGF-I, and leptin. All samples collected after the GnRH challenge (6 samples per bull) were individually assayed for concentrations of testosterone, LH, and FSH.
Analysis was done at the University of Saskatchewan using the following procedures.

Serum concentrations of LH, FSH and leptin were determined by double-antibody radioimmunoassay (RIA), whereas serum concentrations of testosterone and insulin were determined by solid-phase RIA. Serum IGF-I concentrations were determined with a solid-phase, enzyme-labeled, chemiluminescent immunometric assay on a Siemens Immulite 1000 analyser (Siemens AG, Erlangen Germany). Intra- and inter-assay coefficients of variation were < 10% for all hormones. Leptin concentrations were determined using a multi-species leptin RIA kit (Linco Research, St. Charles, MO, USA); the sensitivity of the assay was 1.0 ng/mL.

Testosterone and insulin concentrations were determined using solid-phase RIA kits (Siemens, Los Angeles, CA, USA). Testosterone standards were prepared with purified hormone (Sigma Chemical Co., St. Louis, MO, USA) diluted in charcoal-stripped bovine serum; the sensitivity of the testosterone assay was 0.04 ng/mL. The sensitivity of the insulin assay was 1.2 µIU/mL.

Gonadotropin concentrations were determined as described (Evans et al. 1995). Luteinizing hormone NIH-bLH-B4 [national Hormone and Peptide Program (NHPP), Torrance, CA] was used for preparation of standards and AFP 11743B bLH (NHPP) was used for tracer labelling. Luteinizing hormone (LH) concentrations were determined using rabbit bovine-LH antiserum and the sensitivity of the assay was 0.05 ng/mL. Follicle stimulating hormone AFP5318C (NHPP) was used for preparation of standards and NIDDK-oFSH-I-2 (NHPP) was used for tracer labelling. Follicle-stimulating hormone concentrations were determined using rabbit ovine-FSH antiserum (anti-oFSH-1; AFPC5288113; NHPP) and the sensitivity of the assay was 0.1 ng/mL.
2.4.4 Age at puberty

Once SC reached 26 cm, semen collection was attempted (electroejaculation) every 2 wk to confirm puberty. Puberty was defined as > 50 x 10^6 spermatozoa in the ejaculate with > 10% motility (Wolf et al. 1965); the age at which each bull was confirmed to have reached puberty was recorded. In addition, the age at which each bull first achieved a SC of 28 cm was considered a proxy for puberty, as described (Rawlings et al. 2008).

Bulls were maintained until approximately 72 wk of age and then slaughtered at a local abattoir. Testes were recovered and weighed. Daily sperm production (DSP) per gram of testicular parenchyma (DSP/g) and epididymal sperm reserves (ESR) were estimated from one testis and one epididymis from each bull, as reported (Brito et al. 2007b). In brief, testicular parenchyma samples (approximately 20 g) and separately, the entire epididymis, were thoroughly homogenized and diluted in 0.05% Triton X-100 solution (Sigma). Cells were counted using a hemocytometer and phase-contrast microscopy (400X magnification). Calculations were based on dilutions; DSP/g also used a correction factor of 5.32 d (Amann et al. 1974). Total daily sperm production was calculated by multiplying DSP/g by paired testes weight.

2.4.5 Seminiferous tubular diameter

Testicular tissues were recovered from slaughtered bulls, cut into approximately 4 mm × 4 mm tissue blocks and processed for routine histological evaluation. Briefly tissues were placed in modified Davidson’s fixative, a fixative known to successfully preserve reproductive tissues (Latendresse et al. 2002). Fixative was removed after 24 h and samples were washed in 70% ethanol three times (24 h each). The testes were then embedded in paraaffin blocks. 4 μm sections
were cut and stained with hematoxylin and eosin (H&E). Twenty round tubules from each animal were imaged (100X objective, Leica Microsystems GmbH Wetzlar, Germany). Diameter of tubules were measured using Image J (National Institutes of Health, Bethesda, MD, USA).

2.4.6 Testicular gene expression

2.4.6.1 Isolation of RNA

Total cellular RNA was isolated from testicular tissues using Trizol reagent (Invitrogen, Canada, Burlington, ON, Canada) according to the manufacturer’s recommendations. Total RNA was re-suspended in 100 µL of water and RNA was quantified via spectrophotometry using a NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.4.6.2 Real-Time RT-PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) was conducted as described (Bustin et al. 2009; Klein et al. 2010). Testicular RNA samples (1 µg/reaction) were treated with RNase-free DNase I (Thermo Fisher Scientific) for 15 min at room temperature, 25 mM EDTA was added, followed by heat denaturation (65 °C for 10 min). Reverse transcription was done using High Capacity cDNA Reverse Transcription Kit and random hexamers (Applied Biosystems, Foster City, CA, USA). These testicular samples were evaluated for their expression of 3beta-hydroxysteroid dehydrogenase (3β-HSD), androgen receptor (AR), GATA4, IGF-I, IGF-IR, P27, and Wilms tumor-1 (WT1). Primers (Table 2-1) specific for the selected transcripts were designed using the National Center for Biotechnology and Information (NCBI). Primers were designed to span an exon-exon junction and produce a product < 150 base pairs. Amplification efficiencies were determined to be best for all primer sets using a 1:10 dilution of cDNA samples. Real-time PCR was completed using SYBR Green PCR Master Mix (Applied
Biosystems), with the following cycling conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 59 °C for 1 min, and 55-95 °C for dissociation. All PCR’s were performed on 384 well plates (Invitrogen), with fluids delivered using the epMotion automated pipetting systems (Eppendorf, Westbury, NY, USA). Each PCR was performed in duplicate with non-reverse transcription and no cDNA controls included. Mean threshold cycle (C_q) was determined for each sample to evaluate changes in gene expression and C_q was then normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), producing delta (Δ) C_q values (Klein et al. 2010). The PCR results are displayed as 1/ ΔC_q, to reflect actual expression patterns of genes.

Table 2-1. Primer sequences used to characterize genes in testicular tissues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession #</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>3β-HSD</td>
<td>NM_174343.3</td>
<td>CCACACCAGCACCATAGAAGTG</td>
<td>CTTCTTCACGGCCGTCTTG</td>
<td>71</td>
</tr>
<tr>
<td>AR</td>
<td>NM_001244127.1</td>
<td>GCCCCTGACCTGGTTTTCA</td>
<td>CATCGGACACACTGGCTGTA</td>
<td>69</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_001034034.2</td>
<td>GCATCGTGAGGGACTTATGA</td>
<td>GGGCCATCCACAGTCTTCTG</td>
<td>67</td>
</tr>
<tr>
<td>GATA4</td>
<td>NM_001192877.1</td>
<td>GGGATTTTAGCGCATACCTTTTT</td>
<td>GGCTGAGGTGTGCTCTAGTG</td>
<td>96</td>
</tr>
<tr>
<td>IGF-I</td>
<td>NM_001077828.1</td>
<td>GGTGAAGATGCCCATACATC</td>
<td>GCTGGTGAAAGGCGAGCAA</td>
<td>91</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>XM_010816884.1</td>
<td>TCAAGAGTTATCTCCGTTCTTTGA</td>
<td>CAGGCCCTCTGGATCATTTTG</td>
<td>91</td>
</tr>
<tr>
<td>P27</td>
<td>NM_001100346.1</td>
<td>ACACGCATTGTCGATCAG</td>
<td>AGGGCACTGCTCCGTAAC</td>
<td>68</td>
</tr>
<tr>
<td>WT1</td>
<td>XM_003587002.2</td>
<td>GAGTGCGCGCTCTCTCTCA</td>
<td>CAACCGCGTTTCCTTCTC</td>
<td>73</td>
</tr>
</tbody>
</table>

2.4.7 Statistical analyses

Characteristics of LH secretion (including pulse frequency and pulse amplitude) were determined using PC-Pulsar software (Gitzen and Ramirez, University of Illinois, Chicago, IL, USA). Statistical analyses were conducted using JMP, Version 7 (SAS Institute Inc., Cary, NC,
USA, 1989-2007). A multivariate ANOVA response model was used to determine and locate effects of nutrition (low, medium, high) and age, and the nutrition × age interaction, on BW, SC, PTV, TPI, diameter of the testicular vascular cone, and serum hormone concentrations. If either the main effect or the interaction were significant (Wilks’ Lambda), differences were identified using Tukey’s HSD (Honestly Significant Difference) test. In addition, one-way ANOVA (with Tukey’s HSD) was used to determine and identify diet effects on age at puberty, epididymis weight, DSP/g, ESR, age at > 28 cm SC, paired testes weight, and total DSP. Percentage data were arc-sine transformed prior to analysis (non-transformed data were reported).

Statistical analyses for gene expression was conducted using SPSS Version 22 (IBM SPSS Statistics for Macintosh, Version 22.0., IBM Corp., Armonk, NY, USA). A multivariate ANOVA response model was used to determine and locate effects of nutrition (low and high) on gene expression data. If the data was not normally distributed, a Mann-Whitney U test for independent samples was used. Data is reported as means (± SEM).

2.5 Results

There were nutrition, age and nutrition × age interaction effects (P < 0.0001) on BW. Bulls fed the high-nutrition diet were heavier than those fed the low-nutrition diet from 15 to 71 wk, and heavier than bulls fed the medium-nutrition diet from 19 to 51 wk (Figure 2-1). For SC, there were effects of nutrition, age (P < 0.001 for each) and a nutrition × age interaction (P < 0.006). Bulls fed the high-nutrition diet had larger SC than those fed the low- and medium-nutrition diets from 11 to 71 wk and from 23 to 39 wk, respectively (Figure 2-1). We detected effects of nutrition (P < 0.006), age (P < 0.001) and a nutrition × age interaction (P < 0.001) on PTV. Bulls fed the high-nutrition diet had a greater PTV than those fed the low-nutrition diet
from 15 to 71 wk; furthermore, their PTV exceeded that of the medium-nutrition group at 27 and 39 wk (Figure 2-1). In addition, bulls fed the high-nutrition diet had significantly greater PTV as a percentage of overall BW than bulls fed the low-nutrition diet until 39 wk (Figure 2-2); this trend continued until 71 wk of age (although there was no significant difference between the low- and medium-nutrition diets after 39 wk).
Figure 2-1. Mean (± SEM) for BW, SC, and PTV in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect. Asterisks indicate difference (P < 0.05) among groups within age (*all groups differed; **low differed from high).
We observed age effects (P < 0.0013) and nutrition × age interaction (P < 0.05) on TPI. Bulls fed the high-nutrition diet had significantly greater TPI than those fed the low-nutrition diet at 27 and 31 wk, and significantly lower TPI than those fed the low-nutrition diet at 63 wk (Figure 2-2). There were effects of nutrition (P < 0.05) and age (P < 0.0001) and a nutrition × age interaction (P < 0.002) on mean testicular vascular cone diameter. Bulls fed the high-nutrition diet had larger testicular vascular cone diameters at 11, 27 to 43 and 63 wk compared to bulls fed the low-nutrition diet (Figure 2-2).

Age (P < 0.05) and a nutrition × age interaction (P < 0.05) affected serum LH secretion. All bulls, regardless of nutrition group, had an early rise in LH. However, in bulls fed the high-nutrition diet, this early rise was advanced by 8 wk (11 vs 19 wk) and mean LH concentration was greater compared with that of the other 2 groups (Figure 2-3). The LH pulse frequency had a similar pattern. We observed an age (P < 0.05) effect, with bulls fed the high-nutrition diet having more frequent LH pulses between 11 and 15 wk (Figure 2-3). The peak in LH pulse frequency occurred earlier in the bulls fed the high-nutrition diet compared with the other two groups, and peaked at a higher magnitude than in the bulls fed the low-nutrition diet. There were no significant effects of nutrition or age on LH pulse amplitude (Figure 2-3).

Following the GnRH challenge, we detected effects of age on mean LH and total LH secretion (P < 0.005). In all groups, LH secretion increased following the GnRH challenge at 19 versus 15 wk; this LH secretion peaked from 19 to 23 wk and appeared to plateau at 27 wk (Figure 2-4).
Figure 2-2. Mean (± SEM) PTV as a percentage of total BW, testicular pixel intensity and testicular vascular cone diameter in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect. Asterisks indicate difference (P < 0.05) among groups within age (*all groups differed; **low differed from high).
Figure 2-3. Mean (± SEM) total LH secretion, number of LH pulses and pulse amplitude of LH pulses during 10-h intensive blood sampling (every 15 min) in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect. Within an age, groups without a common letter differed (P < 0.05).
Figure 2-4. Mean (± SEM) serum LH concentrations and total LH during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect.
Effects of age, nutrition and nutrition × age interaction were not significant for basal FSH concentrations or for FSH concentrations after GnRH (Figure 2-5). We detected effects of nutrition (P < 0.05), age (P < 0.05) and a nutrition × age interaction (P < 0.001) on basal serum testosterone concentrations. In all groups, testosterone concentrations increased after 11 wk; bulls fed the high-nutrition diet had greater testosterone concentrations than those fed the low-nutrition diet from 11 to 27 wk (Figure 2-6). There were effects of nutrition (P < 0.05) and age (P < 0.001), and a nutrition × age interaction (P < 0.05) on mean testosterone concentration after GnRH. Bulls fed the high-nutrition diet had a greater response to GnRH (in terms of total testosterone secretion) compared to bulls fed the low-nutrition diet at 15, 19, 23, and 27 wk (Figure 2-6).

We observed nutrition, age and nutrition × age interaction effects (P < 0.0001) on serum IGF-I concentrations; bulls fed the high-nutrition diet had greater IGF-I concentrations than bulls fed either the low- or medium-nutrition diets throughout the entire blood-sampling period (Figure 2-7). However, no nutrition, age or nutrition × age interaction effects were detected for insulin or leptin, (Figure 2-7).

Bulls fed the high-nutrition diet were younger (P < 0.05) at puberty and when they reached a SC of 28 cm than bulls fed the low-nutrition diet. At 72 wk, bulls fed the high-nutrition diet had greater (P < 0.05) paired testes weight, epididymis weight, and ESR than the bulls fed the low-nutrition diet. However, nutrition had no significant effects on age at maturity, DSP/g, or total DSP (Table 2-2).
Figure 2-5. Mean (± SEM) FSH secretion during 10-h intensive blood sampling (every 15 min) and FSH secretion during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect.
Figure 2-6. Mean (± SEM) serum testosterone concentrations during 10-h intensive blood sampling (every 15 min) and during the 90 min after a GnRH challenge in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N×A: nutrition × age interaction effect. Within an age, groups without a common letter differed (P < 0.05).
Figure 2-7. Mean (± SEM) serum concentrations of IGF-I, insulin and leptin during 10-h intensive blood sampling (every 15 min) in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 6 bulls/diet) from 2 to 31 wk of age.

N: nutrition effect; A: age effect; N × A: nutrition × age interaction effect. Within an age, groups without a common letter differed (P < 0.05).
Table 2-2. Mean (± SEM) for various reproductive end points in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th>Item</th>
<th>Low (n = 8)</th>
<th>Medium (n = 9)</th>
<th>High (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at puberty (d)</td>
<td>369.3 ± 14.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>327.4 ± 9.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>324.3 ± 11.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age at SC &gt; 28 cm (d)</td>
<td>371.0 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>335.2 ± 15.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>290.5 ± 12.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paired testes weight (g)</td>
<td>561.6 ± 23.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>611.1 ± 59.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>727 ± 33.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymis weight (g)</td>
<td>27.7 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.5 ± 1.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>33.2 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total DSP (x10&lt;sup&gt;9&lt;/sup&gt; sperm)</td>
<td>6.6 ± 0.9</td>
<td>7.3 ± 0.8</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>DSP/g (x10&lt;sup&gt;6&lt;/sup&gt; sperm/g)</td>
<td>11.6 ± 1.4</td>
<td>12.1 ± 1.0</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>ESR (x10&lt;sup&gt;9&lt;/sup&gt; sperm)</td>
<td>12.7 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.7 ± 1.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.1 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup>Within an end point, means without a common superscripts differed (P < 0.05)

<sup>1</sup>SC = scrotal circumference; DSP = daily sperm production; ESR = epididymal sperm reserves.

Nutrition had no significant effects on seminiferous tubular diameter; tubular diameter (± SEM) was 240.6 ± 1.9, 240.3 ± 1.4 and 244.5 ± 1.3 for the low-, medium-, and high-nutrition groups, respectively. A representative image of a round seminiferous tubule from these adult bulls is shown in Figure 2-8. Nutrition also had no significant effect on expression of WTI (Figure 2-9A), AR (Figure 2-9B), P27 (Figure 2-9D), 3β-HSD (Figure 2-10A), IGF-I (Figure 2-10B) or IGF-IR (Figure 2-10C) in the adult bulls. However, bulls fed the high-nutrition diet had higher expression of GATA4 (P < 0.05; Figure 2-9B) than bulls fed the low-nutrition diet.
Figure 2-8. Representative image of a round seminiferous tubule used to measure seminiferous tubule diameter in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.
Figure 2-9. Mean (± SEM) expression patterns (1/ ΔC_q) of A) *WT1*, B) *AR* C) *GATA4*, and D) *P27* in testes of adult Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.

A,B Diets without a common superscript differed (P < 0.05).
Figure 2-10. Mean (± SEM) expression patterns ($1/\Delta C_q$) of A) $3\beta$-HSD, B) IGF-I and C) IGF-IR in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.
2.6 Discussion

In the present study, bulls fed the high-nutrition diet consistently had larger testes than those fed the low-nutrition diet throughout the entire experimental period. It is noteworthy that these differences in testes size were maintained beyond the differential feeding period, as evident from SC, PTV and paired testes weight (at slaughter). Furthermore, bulls fed the high-nutrition diet were younger at puberty (~45 d) than those fed the low-nutrition diet. An even greater difference (~80 d) between the high- and low-nutrition groups was observed for the age at which SC was > 28 cm (a proxy for puberty; Lunstra et al. 1978). The latter variable is probably a more accurate representation of age at puberty than characteristics of the ejaculate. In that regard, for some bulls, there was a series of ejaculates with no or few sperm, followed by an ejaculate that greatly exceeded the minimum standards; therefore, we inferred that the bulls had previously passed puberty, although we lacked the semen sample to reach the classical definition of puberty (Wolf et al. 1965). Puberty has been reported to occur in Holstein bulls between 39 to 41 wk (Amann 1983). Consistent with this report, bulls fed the high-nutrition diet in the current study attained puberty at a similar age. Earlier puberty, even by 45 d, would be welcomed by the AI industry, as this could hasten collection of semen needed for progeny testing and thus allow bulls to be proof-tested and into production earlier. Overall, these results supported our hypothesis that Holstein bull calves on a high-nutrition diet reach puberty earlier and have larger testes than those on a low-nutrition diet.

Regarding gonadotropin concentrations, we did not characterize the entire prepubertal period, as this has already been done in Holstein and beef bulls (Rawlings et al. 1978; Amann and Walker 1983; Evans et al. 1995; Evans et al. 1996; Brito et al. 2007a). Notwithstanding, we evaluated temporal relationships among gonadotropins, testosterone and metabolic hormones,
during the differential feeding period; in that regard, basic hormone profiles were consistent with the aforementioned reports.

The LH profiles of these Holstein bull calves had a typical early gonadotropin rise, including characteristic increases in basal concentrations and pulse frequency. However, increased LH secretions apparently occurred earlier than in our previous nutrition study in beef bulls (23 wk in the Holstein bulls versus 26 wk in the beef bulls; Brito et al. 2007b; Brito et al. 2007c). This was consistent with a previous report that, on average, Holstein bulls reach puberty earlier than beef bulls (Wolf et al. 1965).

Bulls fed the high-nutrition diet had an earlier and more substantial early rise in LH than those fed the low-nutrition diet, as reported in our beef bull study (Brito et al. 2007a). Consistent with that study, we detected no significant differences in the secretion of gonadotropins following the GnRH challenge, suggesting that diet did not affect the ability of bulls to respond to GnRH. However, there was clear evidence that nutrition influenced the hypothalamus-pituitary-testes axis by modulating the GnRH pulse generator during the early gonadotropin rise (Brito et al. 2007b).

In the present study, bulls fed the high-nutrition diet had increased IGF-I concentrations earlier than the bulls fed the low-nutrition diet. Furthermore, we detected a temporal association between increased IGF-I concentrations and an early LH rise in the bulls fed the high-nutrition diet. Therefore, we inferred that IGF-I had a role in regulating the early gonadotropin rise (in particular, LH) and thus reproductive development of Holstein bulls. Similarly, in previous studies in beef bulls, IGF-I was identified as a possible mediator of the nutritional effect of reproduction on the hypothalamus-pituitary-testes axis (Brito et al. 2007b). In that study, there was a strong association between IGF-I concentrations and BW, SC and PTV. In addition, IGF-I
concentrations were also the single best predictor of BW, back fat, SC and PTV (Brito et al. 2007a). Therefore, IGF-I may be potentiating its effect in two ways: by acting as a messenger of nutritional status to the hypothalamus, and by directly affecting somatic cells of the testes. There are IGF-IRs in GnRH neurons in the hypothalamus, and their numbers increase during sexual development (Daftary and Gore 2004). In addition to the systemic role of IGF-I, it also has important autocrine and paracrine roles at the cellular level, regulating mitosis, apoptosis and cellular differentiation. The testes are clearly a site of IGF-I biosynthesis and action (Wang and Hardy 2004); LH stimulates IGF-I secretion in rodent testes (Cailleau et al. 1990) and both IGF-I and its receptors have been detected in Leydig cells, Sertoli cells and spermatocytes in various species (Hansson et al. 1989; Villalpando et al. 2008). Wang and Hardy (2004) evaluated a target gene deletion of IGF-I in the testes and concluded that both IGF-I and LH were critical factors in determining Leydig cell numbers and their steroidogenic capacity (Wang and Hardy 2004). Furthermore, LH appears to not be a direct mitogenic factor for Leydig cells, but instead, it acts through IGF-I. In that regard, IGF-I had a critical autocrine/paracrine role in control of adult Leydig cell numbers and functioning by promoting both proliferation and differentiation of precursors in the Leydig cell lineage (Wang and Hardy 2004). Furthermore, IGF-I is also critical for Sertoli cell proliferation and function. There was increased Sertoli cell death and decreased proliferation in mouse Sertoli cells with the IGF-IR knocked out (Froment et al. 2007). Additionally, FSH seems to require the IGF signaling pathway to mediate its proliferative effects on Sertoli cells (Froment et al. 2007; Pitetti et al. 2013). This further substantiated our hormone data, suggesting that both LH and IGF-I affected the testicular environment by promoting testicular development, perhaps as a consequence of an increasing somatic cell population; namely, promoting proliferation, differentiation or both, of these somatic cells. Furthermore, in
another study, IGF-I treatment increased “gonadal sensitivity to gonadotropins and hastened puberty in female monkeys” (Wilson 1998). In rats, exogenous IGF-I significantly reduced age at puberty and stimulated GnRH release from the hypothalamus (Hiney et al. 1996). In the present study, bulls fed the high-nutrition diet had more LH, and IGF-I increased earlier; based on the reports cited above, these differences presumably contributed to the observed increase in testicular size.

It is noteworthy that IGF-I up-regulated secretion of testosterone from Leydig cells, consistent with a previous report (Lin et al. 1986) and indicative of a positive feedback. In that regard, IGF-I initially up-regulates testosterone production from Leydig cells, which up-regulated both the IGF-IR and IGF-I production by Leydig cells. In the present study, testosterone concentrations increased steadily from 11 to 31 wk, consistent with previous reports (Amann and Walker 1983). That testosterone was also affected by nutrition modulation supports the assertion that the higher level of nutrition hastened reproductive development in the bulls fed the high-nutrition diet. These testosterone data were consistent with the conclusions that nutritional modulation affected LH and IGF-I concentrations and thus testicular somatic cells. In a previous study, effects of IGF-I deletion on development of the adult Leydig cell population were assessed by evaluating effects on Leydig cell precursor cells in mice (Hu et al. 2010). In that study, decreased concentrations of testicular testosterone in IGF null mice from postnatal days 21 to 90 were attributed to the absence of IGF-I.

In the present study, TPI increased from 23 to 39 wk, with TPI increasing earliest in bulls fed the high-nutrition diet. This was consistent with previous reports, based on increased testicular echogenicity (Brito et al. 2012), which is apparently associated with maturation of the testes, including formation of the blood testes barrier, Sertoli cell differentiation and ultimately
initiation of spermatogenesis (Curtis and Amann 1981; Wrobel 1990). However, there was a slightly different pattern of TPI in this study; following the characteristic increase, there was a much larger decrease in TPI in Holstein bulls, compared to beef bulls (Brito et al. 2012). Although TPI was useful for determining puberty and maturation status, it has inconsistent associations with sperm production, seminiferous tubule area, epithelium area, and sperm morphology following bull maturation (Brito et al. 2012). Therefore, TPI may be of less value in post-pubertal bulls.

The values of DSP/g were lower in the bulls fed the high-nutrition diet versus bulls fed the low-nutrition diet; consequently, there were no significant difference in total DSP among the three diets, which was unexpected. Notwithstanding, there was a trend for bulls fed the high-nutrition diet to have to have a greater total DSP. In the previous beef bull study, DSP/g values were numerically higher in the bulls fed the high-nutrition diet, and the total DSP was significantly higher in this group, compared to those fed the low-nutrition diet (Brito et al. 2007c). We may have failed to detect this significance due to breed differences. The ESR for bulls fed the high-nutrition diet exceeded those of the bulls fed the low-nutrition diet, and the high-nutrition bulls also had greater epididymis weights at slaughter, consistent with their larger testes.

There was no significant difference among diets in seminiferous tubular diameter of the Holstein bulls. Based on these results, together with the DSP and DSP/g results, it was concluded that Sertoli cell density (per unit of testicular tissue or cross-sectional area) was not altered. Therefore, early-life nutritional modulation did not affect the efficiency of spermatogenesis (number of spermatozoa produced per gram of testicular parenchyma, mainly influenced by Sertoli cell numbers), a parameter known to be relatively constant among bulls (Macmillan and
Hafs 1968; Killian and Amann 1972; Lunstra and Cundiff 2003; Brito 2014). Therefore, we inferred that more sperm produced in bulls receiving high-nutrition diets must be due to more total cells present in the testes, leading to longer seminiferous tubules or more tubules, rather than larger tubules.

The genes *WT1, GATA4, AR* and *P27* are well-established Sertoli cell markers (Sharpe *et al.* 2003). In rodents, *WT1* is turned on early in life and is then continuously expressed in Sertoli cells (Sharpe *et al.* 2003). Furthermore, *AR*, is known to be expressed in mature Sertoli cells, but does first appear prior to final maturation (Sharpe *et al.* 2003). Expression of *P27* in Sertoli cells coincides with their maturation, as its expression is associated with inhibition of proliferation (Sharpe *et al.* 2003). Expression of *GATA4* in the testes is activated very early, remains abundant in adult tests (Chen *et al.* 2015), and is required for proper testes development, regulation of steroidogenesis and adult Sertoli cell function in rodents (Mazaud Guittot *et al.* 2007; Kyronlahti *et al.* 2011; Chen *et al.* 2015). Sertoli cell specific knockout in adult mice for *GATA4* caused impaired fertility and Sertoli cell dysfunction (Kyronlahti *et al.* 2011). In the present study, there was a significantly higher level of *GATA4* expression in the high-nutrition bulls compared to low-nutrition bulls, which was inconsistent with the other Sertoli cell markers measured. However, it is interesting to note that there was an overall trend for all markers measured here to have higher expression in high- versus low-nutrition bulls.

Overall, the present data were consistent with our previous work in beef bulls and provided clear evidence that nutritional modulation of Holstein bull calves during early-life had profound effects on reproductive development. It was unclear, however, whether nutritional modulation is required for the entire 31 wk to obtain these effects on reproductive development, or if a shorter interval would be as effective. Furthermore, the present findings supported the
conclusion that because the beneficial effects of enhanced nutrition extended beyond the differential feeding period and into adulthood, these effects were probably related to increased LH concentrations during early calfhood (Brito et al. 2007c). Overall, feeding Holstein bull calves diets higher than recommended levels of energy and protein during early-life (2 to 31 wk) resulted in bulls that reached puberty earlier and had larger testes than those that were underfed. These findings have important implications for management of young dairy bulls before their arrival at AI centers. Hastening puberty allows earlier collection of semen for bull progeny testing, enabling AI centers to determine earlier which bulls are the most valuable to them, and thus minimize costs associated with housing and maintaining unwanted bulls for longer intervals. In addition, producing bulls with larger testes results in more semen doses being produced, and thus should increase profit. Moreover, the results of this study supported nutritional modulation as a model for studying mechanisms underlying the control of reproductive development in bulls.

Based on the results described in this chapter, I hypothesized that it is the higher concentrations of IGF-I in the high-nutrition bull calves that are responsible for stimulating Sertoli cells to proliferate and differentiate earlier and more extensively than the low-nutrition calves, leading to larger testes (investigated in the following chapters).

**2.7 Acknowledgements**

This work was supported by the Canadian Agriculture Adaptation Program (Ottawa, ON, Canada) and L'Alliance Boviteq Inc (Saint-Hyacinthe, QC, Canada).
Chapter Three: **Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing post-pubertal semen quality**

### 3.1 Preamble

This study is in press in *Theriogenology*

Title: Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing post-pubertal semen quality, Theriogenology (in press, April 2016).

Alysha Dance, Jacob Thundathil, Patrick Blondin, and John Kastelic

A.D preformed 95% of experiments, analyzed 95% of the results and contributed 95% of the writing. J.T. supervised the project and reviewed the manuscript, P. B. provided industry support for the project and reviewed the manuscript and J. K. co-supervised the project and reviewed the manuscript.

### 3.2 Abstract

Enhanced early-life nutrition (~130% of required energy and protein) increased testes size and weight (~20-25%) and reduced age at puberty (~1 mo) in beef and dairy bulls, compared to those fed 70% of dietary requirements. The objective was to determine effects of early-life (2 to 31 wk) nutritional modulation on feed costs, predicted number of harvestable sperm and doses of semen, and semen quality. Calves (~1 wk old) were randomly allocated into three groups that were fed 4, 6, or 8 L/d of milk [low (n = 8), medium (n = 9) and high groups (n = 9), respectively] from 2 to 8 wk of age. Thereafter, they were weaned, transitioned onto barley
silage-based diets in preparation to receive ~70, 100, or 130% of recommended amounts of energy and protein (feed costs were ~CAD$280 more per bull to feed high versus low diets from 2 to 31 wk). After 31 wk, all bulls were fed a medium diet. Semen was collected, by electroejaculation, from 51 to 73 wk, extended, chilled, and cryopreserved. Bulls fed high-nutrition were numerically younger (P = 0.45) at sexual maturity (sperm with ≥30% progressive motility; ≥70% morphologically normal, and ≤20% abnormal heads), first acceptable post-chill sperm motility (> 50%; P = 0.66) and first acceptable post-thaw motility (> 25% progressive; P = 0.25) than bulls in the low-nutrition group. Semen from three bulls per group was used for *in vitro* fertilization (total of 1249 bovine oocytes); there were no significant differences among groups in fertilization percentage (mean ± SEM of 68.0 ± 8.7, 77.1 ± 3.5 and 68.7 ± 4.5% for low, medium and high, respectively) or blastocyst yield (31.5 ± 5.6, 41.4 ± 4.9 and 33.7 ± 4.6%). Based on analysis of 2D gels of sperm proteins, 380 spots were identified on the fused master gel, but no spots were differentially expressed across groups. Overall, there were no significant differences in semen quality or sperm function among bulls fed three levels of nutrition from 2 to 31 wk of age. However, we estimated that bulls fed high-nutrition early in life had the potential to produce more sperm resulting in ~$2200 more per collection compared to a low-nutrition bull. This increase in profitability with no changes in semen quality, supports enhanced early-life nutrition as a management tool to improve reproductive potential of dairy bulls.

**Key words:** Sperm quality; diet; fertility; male reproductive performance; IVF
3.3 Introduction

We recently reported (Dance et al. 2015) that enhanced early-life nutrition, both energy and protein, from 2 to 31 wk of age, resulted in bulls that were ~ 45 d younger at puberty ($\geq 50 \times 10^6$ sperm per ejaculate; Wolf et al. 1965) and they were also ~ 80 d younger when their SC reached 28 cm, a proxy for puberty (Rawlings et al. 2008), compared to bulls that were underfed from 2 to 31 wk. Furthermore, testis weight was increased by ~20-25% at 74 wk of age in well-fed bulls. In another recent study (Harstine et al. 2015), increased early-life nutrition in bulls increased testes size. Larger testes are expected to increase the number of harvestable sperm, as efficiency of spermatogenesis, namely the number of sperm produced per gram of testicular parenchyma, is relatively constant among bulls (10-14 $\times 10^6$ sperm/g parenchyma; Macmillan and Hafs 1968; Killian and Amann 1972; Lunstra and Cundiff 2003; Brito 2014) and therefore, daily sperm production is determined by paired testes weight (Brito 2014).

It is well established that overfeeding bulls after puberty can reduce reproductive performance (Young 1974; Brown 1994); this is attributed to increased fat in the scrotal stem, which presumably interferes with testicular thermoregulation, thereby increasing testicular temperature and decreasing semen quality (Brown 1994). Although enhanced early-life nutrition increased testicular size and hastened puberty in our previous studies, its effects on post-pubertal semen quality have not been well characterized. In one study (Brito 2006), bulls fed various diets from 11 to 31 wk, sperm morphology did not differ significantly once bulls reached sexual maturity. In another study (Flipse and Almquist 1961), dairy bulls were fed various levels of energy from early-life to 4 y of age. Although the low-nutrition group at 80 wk temporarily had lower average motility than the other groups, this difference disappeared as the bulls matured.
With increasing implementation of genomic selection in choosing future AI sires, there is a desire to start collecting and marketing semen from genetically superior bulls earlier in their life. Therefore, bulls that become sexually mature and produce freezable semen younger are favored. Furthermore, the number of insemination doses that can be produced and sold affect profitability. We reported benefits of increased early-life nutrition, with high-nutrition bulls having significantly larger testes at an earlier age than low-nutrition calves (Dance et al. 2015). However, for the industry to adopt increased early-life nutrition in bulls, cost-benefits and consequences of this management on semen quality should be determined. Therefore, the objective was to determine effects of early-life (2 to 31 wk) nutritional modulation on feed costs, estimated sperm available for harvest, estimated number of insemination doses, and semen quality, including sperm morphology, motility, sperm proteins, fertility, and viability. We tested the hypothesis that varying levels of nutrition from 2 to 31 wk does not significantly affect sperm motility, morphology or fertility in a homologous in vitro fertilization system, but increases estimated number of insemination doses.

3.4 Materials and methods

3.4.1 Bulls and treatments

Twenty-six Holstein bull calves were used; these were the same bulls used in a previous report from our laboratory (Dance et al. 2015). In brief, calves were randomly allocated into one of three groups at approximately 1 wk of age to receive a low, medium, or high diet, from 2 to 31 wk of age [low (n = 8), medium (n = 9), and high (n = 9)]. Calves were randomly allocated into groups so that average body weights of the three groups were similar. Unfortunately, birth weight data were not available, as calves were born on various dairy farms and soon thereafter,
brought to a calf-rearing operation, where they were maintained until weaning. Calves were fed high-quality, reconstituted milk replacer (4, 6 or 8 L/d in low, medium and high groups, respectively), from 2 to 8 wk of age, and then transitioned onto barley silage-based diets, as described (Dance et al. 2015). Calves were fed differential diets until 31 wk, and thereafter, all were maintained on the medium diet. This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the Lethbridge Research Centre Institutional Animal Care Committee.

3.4.2 Cost of differential feeding

Costs of the three diets were estimated based on current market values of feed ingredients. Calves were fed three levels of milk from 2 to 8 wk of age and then transitioned onto a silage-based total mixed ration. We estimated milk consumption given that they were on either 4, 6 or 8 L/d (low, medium and high respectively). The current cost of milk replacer in western Canada (CAD$4.65/kg) was used to calculate the cost of the milk portion of the diets (Table 3-1). After 8 wk, calves were transitioned on to a silage-based diet. Feed costs during transition (8 to 11 wk) were assumed to be similar among groups and were excluded from current calculations.
Table 3-1. Diet costs (CAD$) for Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total milk replacer (L/bull; 2 to 8 wk)</td>
<td>168</td>
<td>252</td>
<td>336</td>
</tr>
<tr>
<td>Cost of milk replacer/bull</td>
<td>$97.65</td>
<td>$146.48</td>
<td>$195.30</td>
</tr>
<tr>
<td>Total feed/bull (kg, as-fed; 11 to 31 wk)</td>
<td>1248.2</td>
<td>1420.6</td>
<td>533.1</td>
</tr>
<tr>
<td>Cost of diet/bull (11 to 31 wk)</td>
<td>$156.21</td>
<td>$245.20</td>
<td>$337.59</td>
</tr>
<tr>
<td>Total cost of feeding</td>
<td>$253.86</td>
<td>$391.67</td>
<td>$532.89</td>
</tr>
</tbody>
</table>

Feed intakes per pen were recorded on a daily basis from 11 to 31 wk of age. The sum of all feed intakes combined for all calves in a nutrition group were calculated and then divided by the number of calves in that group, yielding total feed consumed on a per bull basis from 11 to 31 wk. Diet composition has been reported (Dance et al. 2015). In brief, all diets contained 1.6% vitamin-mineral premix (as-fed). The low-nutrition diet group (n = 8) received barley silage (plus premix, but no concentrate). The medium-nutrition diet group (n = 9) received barley silage plus 4.8% rolled barley, 4.8% rolled corn, 3.8% canola meal, and 3.8% soybean meal, whereas the high-nutrition diet group (n = 9) received barley silage plus 9.7% rolled barley, 9.7% rolled corn, 7.6% canola meal, and 7.6% soybean meal. Using market prices in western Canada (Table 3-2), the cost of the three diets for the entire differential feeding period (11 to 31 wk) per calf was determined (Table 3-1).
Table 3-2. Costs of feed ingredients (December 2015) used for calculation of total diet costs.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Cost (CAD$/tonne)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley silage</td>
<td>60</td>
</tr>
<tr>
<td>Rolled barley</td>
<td>230</td>
</tr>
<tr>
<td>Rolled corn</td>
<td>265</td>
</tr>
<tr>
<td>Canola meal</td>
<td>345</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>575</td>
</tr>
<tr>
<td>Vit/Min Pre-mix</td>
<td>4000</td>
</tr>
</tbody>
</table>

3.4.3 Sexual development and testicular characteristics

Body weight and testicular growth characteristics of these bulls were measured monthly and have been reported (Dance et al. 2015). Once SC reached 26 cm, semen collection via electroejaculation was attempted every 2 wk to confirm puberty. Age at puberty (> 50 x 10^6 sperm of which > 10% were motile; Wolf et al. 1965) and age at which SC reached 28 cm (proxy for puberty (Rawlings et al. 2008) were also reported (Dance et al. 2015).

3.4.4 Age at maturity

Semen was collected, by electroejaculation, every 2 wk after confirmation of puberty. A standard electroejaculator (model BS-1, Bonded Electro, San Diego, CA, USA) with a rectal probe, approximately 7 cm in diameter and 40 cm long, with three ventral electrodes, was used. The electroejaculator was operated by a highly experienced technician. A recurrent cycle of approximately 4 to 6 s of stimulation, followed by 3 to 4 s of no stimulation was used, with a gradual increase, over time, in stimulation intensity. This continued until a sample was collected, but stopped after no more than 2 min (in the absence of a sample). In prepubertal bulls, only a
single round of stimulation was used; if no sample was collected, the bull was allowed to exit the chute, and the procedure repeated in 2 wk. However, in post-pubertal bulls, if the first round of stimulation failed to yield an apparently acceptable ejaculate (based on volume, visual assessment of sperm concentration and subjective microscopic assessment of sperm motility), the bull was allowed to rest for approximately 5 min, and a second round of stimulation was applied (which was stopped upon collection of an apparently satisfactory sample, or after 2 min, whichever occurred first). On any day, a maximum of two rounds of stimulation were applied.

Semen smears were stained with eosin-nigrosin and examined under oil immersion (1000X) using a Leica DM 2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany). Spermatozoa were classified based on morphology, as described (Barth and Oko 1989). Changes in percentage normal as bulls aged are shown in Figure 3-2. Age of maturity was defined as the first time a bull had ≥ 30% progressively motile sperm, ≥ 70% morphologically normal sperm and ≤ 20% sperm with morphologically abnormal heads (Barth and Oko 1989).

### 3.4.5 Incremental value of increased sperm production

In bulls, average daily sperm production per gram of testicular parenchyma is 10-14 x 10^6 sperm (Macmillan and Hafs 1968; Killian and Amann 1972; Lunstra and Cundiff 2003; Brito 2014). Therefore, based on the low end of this range (10 x 10^6 sperm/g/d) and paired testis weight at slaughter (Dance et al. 2015), we estimated the number of sperm each nutrition group produced per day (Table 3-3) once they were sexually mature and in a typical AI setting. We calculated the incremental value of increased sperm production based on just one collection. We assumed that: fixed costs of housing bulls and costs of semen collection would not be affected by early-life nutrition; the incremental cost of freezing one additional dose of semen we used was
CAD$0.25 per dose; the average value of a commercial dose of Holstein bull semen was CAD$20; and each straw would contain $15 \times 10^6$ sperm. Regarding the latter, $15 \times 10^6$ spermatozoa is the average number of sperm in an insemination dose in Canada and is in the middle of the range used in the USA (Vishwanath 2003). Additionally, CAD$20/insemination dose is consistent with the average price of a semen dose in western Canada and globally (Vishwanath 2003).

### 3.4.6 Semen freezing

Once a bull was confirmed to have reached age of maturity, semen freezing was attempted. Samples were collected by electroejaculation and after initial examination for motility and sample quality, semen was diluted with semen extender (Andromed®, Minitube, Ingersoll, ON, Canada) and placed in a 15 mL centrifuge tube, which was placed into a 250 mL glass beaker with ~ 200 mL warm water (37 °C) and that beaker was placed at 4 °C for at least 4 h for chilling and equilibration (Krishnakumar et al. 2011). Then, an aliquot was examined (subjective visual assessment using 200X magnification), and samples subjectively deemed to have > 50% motility were considered acceptable to continue with semen freezing; the age at which each bull met this criterion was designated age at first acceptable chill. Sperm concentration was determined using a hemocytometer, and adjusted (by adding chilled extender) to 50 x $10^6$ sperm/mL. Then, chilled extended semen was loaded into 0.5 mL straws using a semi-automatic semen straw filler (Minitube) in a cold cabinet (4 °C), and straws sealed with glass beads (Minitube). Straws were then frozen using the bovine semen curve on the automatic freezing machine (ICE Cube, Minitube) and plunged into LN2. Semen was frozen for a minimum of 3 d and then thawed (37 °C for at least 30 s). For each bull, the age at which they produced their first
sample with acceptable post-thaw motility (defined as > 25% progressive motility), based on computer-assisted sperm analysis (CASA; Sperm VISION 3.5 software; Minitube) was recorded. However, since some bulls never produced acceptable post-thaw semen (> 25% progressively motile cells), only samples from the following number of bulls in each treatment are reported: low (n = 5), medium (n = 6) and high (n = 8).

3.4.7 Evaluation of motion characteristics of post-thaw sperm

Motility of post-thaw sperm was objectively evaluated by CASA, as described (Krishnakumar et al. 2013). Briefly, a 4-μL aliquot of semen was loaded into a pre-warmed chamber slide (20 μm deep; Leja Products, Nieuw-109, Vennep, The Netherlands) and immediately analyzed under 200X magnification, and total and progressive sperm motility on the first freezable sample from each bull were recorded. Seven microscopic fields per sample were analyzed and the frame rate used was 60/s.

3.4.8 Evaluation of in vitro fertilizing ability

Fertilizing ability was evaluated using in vitro fertilization (IVF) and embryo culture. A subset (n = 3) of bulls from each nutrition group, with a pooled sample of three ejaculates per bull, was used for IVF. The average age of the bulls when these samples were collected was 64.8 ± 1.2 wk (mean ± SEM), with no significant difference in age among treatment groups.

In vitro fertilization was conducted, as reported (Krishnakumar et al. 2011). In brief, oocytes were aspirated from abattoir-derived bovine ovaries and were in vitro matured (IVM) for 22 h at 39 °C and 5% CO2. IVM media comprised of TCM199 supplemented with 25 μM sodium pyruvate, 25 μg/mL gentamicin, 0.5 μg/mL FSH, 5 μg/mL LH, 2 μg/mL estradiol-17β,
and 10% fetal calf serum (Hycolone, Logan, UT, USA). Mature cumulus oocyte complexes (COC) were moved to drops of FERT-TALP (10 oocytes per drop (Parrish et al. 1986), supplemented with 6 mg/mL fatty acid-free bovine serum albumin, 100 units/mL penicillin, 100 μg/mL streptomycin, 25 μM sodium pyruvate, 20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine, and 5 μg/mL heparin.

Sperm were thawed for 1 min in a 37 °C water bath, washed by centrifugation (500 x g, 5 min), re-suspended in the FERT-TALP media, and then washed once more with FERT-TALP, to ensure removal of extender. The resulting sperm pellet was overlaid with 200 μL FERT-TALP and motile sperm were allowed to swim-up for 15 to 20 min at 39 °C and 5% CO2. The upper two-thirds of the media was collected, sperm concentration was determined using a hemocytometer, and used to inseminate oocytes (final concentration in oocyte drops = 1 x 10⁶ sperm/mL). Oocytes were co-incubated with sperm for 18 h. Following co-incubation, presumptive zygotes were denuded of their cumulus cells by gentle vortexing. They were then cultured in 40 μL drops (20 presumptive zygotes per drop) of synthetic oviduct fluid (SOF; 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH₂PO₄, 25.06 mM NaHCO₃, 0.3 mM sodium pyruvate, 2.5 mM sodium lactate 60% syrup, 1 mM glutamine, 8 mg/mL bovine serum albumin, 1 X BME essential amino acids, and 1 X MEM non-essential amino-acids) for 8 d. Fertilizing ability was determined based on cleavage percentage recorded 48 h after fertilization. Embryos were evaluated every 48 h, until 8 d after fertilization, and development to the blastocyst stage recorded.
3.4.9 *Sperm viability*

The same three ejaculates from the same three bulls per treatment group, as used for IVF, were pooled and used to assess sperm plasma membrane viability, as described (Shojaei *et al.* 2012). Briefly, viability was assessed using the SYBR14 and propidium iodide (PI) from the Live/Dead Sperm Kit (Invitrogen). Spermatozoa from each bull and ejaculate were thawed for 1 min in a 37 °C water bath, washed by centrifugation (500 x g, 10 min) to remove extender and washed two more times with PBS (500 x g, 10 min). Sperm were re-suspended in 1 mL PBS and stained with SYBR14 (final concentration, 100 nM) at room temperature for 10 min followed by PI (final concentration, 24 μM) added 5 min before analysis. The SYBR14 stains nuclei of viable cells green, whereas PI stains sperm that are dead (or have a compromised plasma membrane) red. Samples were subsequently analyzed using flow cytometry (FACScan Beckon Dickinson, San Jose, CA, USA). Spermatozoa that emitted both green and red fluorescence were considered moribund. Figure 3-1 shows the gating used to quantify these populations. A minimum of 2 x 10^4 events were recorded for each sample, and each sample was analyzed on three independent occasions (the average of all three replicates was reported). The rate of conversion of live to moribund sperm was determined by expressing moribund sperm as a percentage of total number of moribund and viable sperm.
Figure 3-1. Example flow cytometry output for sperm stained with SYBR14 and PI to evaluate viability.
A) represents the gating to establish the population; B) shows the four population of cells; C) histogram for PI; and D) Histogram for SYBR14 E) shows the percentages of each population.
3.4.10 Two-dimensional (2D) gel electrophoresis (sperm proteins)

Expression patterns of sperm proteins were compared among treatment groups by two-dimensional gel electrophoresis, as described (Shojaei Saadi et al. 2013). For this, semen was collected from a subset of the bulls (n = 3 per group, when bulls were approximately 67 and 71 wk old) and a small volume of the raw ejaculate was added to 1 mL of 0.25% gluteraldehyde solution to determine sperm concentration. Volume of the ejaculate was also recorded. Samples were then centrifuged (700 x g for 10 min) to remove seminal plasma, the resulting sperm pellet was then washed (twice) in TALPH (with protease inhibitor), snap-frozen on dry ice, and stored at -80 °C until used. Two ejaculates per bull, and two replicates of each ejaculate (using the same protein samples) were subjected to gel electrophoresis (to account for ejaculate and technical variations). The remainder of the 2D gel protocol was as described (Shojaei Saadi et al. 2013). Briefly, total sperm proteins were extracted in 2D sample buffer (8.0 M urea, 2.0 M thiourea, 20 mM dithiothreitol (DTT), 1.2 mM tributylphosphine (TBP), 4% w/v CHAPS, and 1X protease inhibitor cocktail) and rehydrated onto an immobilized pH gradient (IPG) strip. Proteins were focused based on their isoelectric point, and then run on a SDS-PAGE gel. To minimize technical variations, four gels were cast and run simultaneously. Following electrophoresis, gels were fixed, stained with SyproRuby (Bio-Rad, Mississauga, ON, Canada) overnight and then imaged with a ChemiDoc XRS+ molecular imager (Bio-Rad).

3.4.11 Protein profile analysis

To compare protein profiles of sperm among all three nutrition groups, gel images were compared and analyzed using gel analysis software (Delta2D 4.0, Decodon, Greifswald, Germany). To enable quantitative comparative spot analysis, normalization occurred at two
stages: first, at 2D-PAGE by loading a consistent amount of protein, and second, at analysis using Delta2D by expressing normalized spot volumes as a fraction of the sum of all spot volumes in the gel (Butt et al. 2006). Evaluation of differentially expressed protein spots used the following strict criteria: (i) significant difference in protein expression (Wilcoxon Rank Sum Test, \( P < 0.05 \)); (ii) 100% reproducibility (coefficient of variation = \( \sigma/\mu < 1 \)) across the sample population; and (iii) False Discovery Rate (FDR) < 0.01 (Hunt et al. 2005).

3.4.12 Statistical analyses

Statistical analyses were conducted using JMP, Version 7 (SAS Institute Inc., Cary, NC, USA). One-way ANOVA (with Tukey’s HSD) was used to determine and locate effects of diet on age at maturity, age at first acceptable chill, age at first freezable sample, motility end points, and IVF results. Percentage data were arc-sine transformed prior to analysis (non-transformed data were reported). Data were reported as mean ± SEM.

3.5 Results

High-nutrition calves consumed more milk replacer (additional costs of CAD$97.70 per bull compared to low-nutrition calves (Table 3-1). During the differential feeding period after weaning (11 to 31 wk), high-nutrition calves each consumed 284.9 kg more feed (value, CAD$181.40) than low-nutrition calves. Overall, feeding each high-nutrition calf from 2 to 31 wk cost CAD$279.03 more than feeding each low-nutrition calf (Table 3-1).

Estimates of daily sperm production were \( 7.270 \times 10^9 \) and \( 5.616 \times 10^9 \) for bulls that had been raised on high- versus low early-life nutrition, respectively (Table 3-3). We estimated that this would result in 485 doses being frozen from each high-nutrition bull per collection versus
374 doses from each low-nutrition bull, with a gross return of CAD$9700 and CAD$7480 respectively (Table 3-3).

Table 3-3. Estimates of total sperm produced per day, number of resulting AI doses and the value of those doses from Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm produced per day (x 10^6)</td>
<td>5616</td>
<td>6111</td>
<td>7270</td>
</tr>
<tr>
<td>No. AI doses</td>
<td>374</td>
<td>407</td>
<td>485</td>
</tr>
<tr>
<td>Value of semen doses (CAD$)</td>
<td>$7480</td>
<td>$8140</td>
<td>$9700</td>
</tr>
</tbody>
</table>

Bulls fed the high-nutrition diet were numerically younger at maturity, age at first chill and age at first post-thaw, than bulls in the low-nutrition group (Table 3-4), although differences were not significant (P = 0.45, P = 0.66 and P = 0.25 respectively). For each bull, percentage of morphologically normal sperm over time are shown in Figure 3-2.
Figure 3-2. Percentage of morphologically normal sperm over time in Holstein bulls fed low-, medium-, or high-nutrition diets (top, middle and bottom panels respectively) from 2 to 31 wk of age.

Each line represents one bull.
Table 3-4. Mean (± SEM) ages (d) for various reproductive end points and progressive motility (%) at first freezable semen samples in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 8 or 9 bulls/diet) from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th>End Point</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturity (d)</td>
<td>385.0 ± 15.7</td>
<td>391.2 ± 12.6</td>
<td>365.6 ± 16.7</td>
</tr>
<tr>
<td>First chill (d)</td>
<td>413.0 ± 15.3</td>
<td>413.8 ± 10.7</td>
<td>397.4 ± 17.1</td>
</tr>
<tr>
<td>First post-thaw (d)</td>
<td>425.6 ± 13.6</td>
<td>457.3 ± 12.8</td>
<td>421.8 ± 14.3</td>
</tr>
<tr>
<td>Progressive motility at 1st freezable semen sample</td>
<td>36.4 ± 4.0</td>
<td>32.1 ± 3.9</td>
<td>36.0 ± 2.1</td>
</tr>
</tbody>
</table>

Note: No significant effect of group for any end point.

Definitions:
- Age of maturity: first time sperm had ≥ 30% progressively motility, ≥ 70% morphologically normal and ≤ 20% morphologically abnormal heads;
- Age of first chill: > 50% motility
- Age of first post-thaw: first sample > 25% post-thaw progressive motility (CASA)

A total of 1249 oocytes were used for IVF. Fertilization percentages were based on the number of oocytes cleaved in each group on Day 2 (Day 0 = fertilization). There were no significant differences among groups in fertilization percentage or blastocyst yield (Table 3-5).
Table 3-5. Mean (± SEM) end points for in vitro fertilization using sperm from Holstein bulls fed low-, medium-, or high-nutrition diets from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th>IVF end points</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cultured</td>
<td>419</td>
<td>420</td>
<td>410</td>
</tr>
<tr>
<td>No. cleaved</td>
<td>285</td>
<td>324</td>
<td>282</td>
</tr>
<tr>
<td>Cleaved (%)</td>
<td>68.0 ± 8.7</td>
<td>77.1 ± 3.5</td>
<td>68.7 ± 4.5</td>
</tr>
<tr>
<td>No. blastocysts</td>
<td>132</td>
<td>174</td>
<td>138</td>
</tr>
<tr>
<td>Blastocysts (%)</td>
<td>31.5 ± 5.6</td>
<td>41.4 ± 4.9</td>
<td>33.7 ± 4.6</td>
</tr>
</tbody>
</table>

No difference (P > 0.05) among groups for any end point.

Semen used was from three bulls from each nutrition group, with a pooled sample of three ejaculates per bull. Mean ± SEM age at sample collection was 64.8 ± 1.2 wk (no significant difference among treatment groups).

The high-nutrition group had significantly more dead sperm than the other two nutrition groups. However, there were no other significant differences among groups regarding percentages of live or moribund sperm (Table 3-6).
Table 3-6. Mean (± SEM) viability results from Live/Dead sperm kit using flow cytometry in Holstein bulls fed low-, medium-, or high-nutrition diets (n = 3 bulls/diet; three ejaculates per bull) from 2 to 31 wk of age.

<table>
<thead>
<tr>
<th>Viability end points (%)</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR14 Positive (live)</td>
<td>57.0 ± 5.9</td>
<td>55.2 ± 6.7</td>
<td>52.6 ± 6.5</td>
</tr>
<tr>
<td>PI Positive (dead)</td>
<td>20.3 ± 1.9a</td>
<td>23.8 ± 4.0a</td>
<td>30.9 ± 4.7b</td>
</tr>
<tr>
<td>Moribund</td>
<td>20.9 ± 7.6</td>
<td>19.0 ± 3.8</td>
<td>15.9 ± 1.7</td>
</tr>
<tr>
<td>Moribund/total viable</td>
<td>26.4 ± 9.0</td>
<td>25.6 ± 6.1</td>
<td>22.1 ± 4.2</td>
</tr>
</tbody>
</table>

a,bWithin a row, means without a common superscript differed (P < 0.05).

Based on analysis of the 2D gels from bulls from the three nutrition groups, 380 spots were identified on the fused master gel. However, no spots were differentially expressed spots across treatment groups. A representative gel is shown (Figure 3-3), with one gel from each nutrition group also available (Figure 3-4).
Figure 3-3. Representative 2D gel of sperm proteins from Holstein bulls fed low-, medium-, or high-nutrition diets (n = 3 bulls/diet) from 2 to 31 wk of age.

There were no significant differences among groups.
Figure 3-4. Representative 2D-gel of sperm proteins from Holstein bulls fed low-, medium-, or high-nutrition diets (left to right, n = 3 per group) from 2 to 31 wk of age.

There were no significant differences among groups.

3.6 Discussion

We recently reported that increased early-life nutrition (2 to 31 wk) decreased age at puberty and increased testes size (Dance et al. 2015). In the current study, we investigated the effects of early-life nutrition (2 to 31 wk) on costs of differential feed intake, expected increase in harvestable sperm, and effects on post-pubertal semen quality and sperm function of Holstein bulls. Increased early-life nutrition had a very modest cost, but generated a large increase in harvestable sperm. Most importantly, semen quality was not significantly different among bulls fed three levels of nutrition from 2 to 31 wk of age.

We estimated that bulls in the high-nutrition group would produce ~9% more sperm per day than the bulls in the medium-nutrition group and ~30% more than the bulls in the low-nutrition group. If all sperm produced could be harvested, and based on an incremental cost of CAD$0.25 for each additional straw that was cryopreserved and a nominal value of CAD$20 per
straw, the net increase in profits would be CAD$2176 per collection for a high-nutrition bull compared to a low-nutrition bull and CAD$1526 for a high-nutrition bull compared to a medium-nutrition bull per collection. In practice, it seems unlikely that all of these sperm will actually be collected. Notwithstanding, these calculations illustrated the principle that the larger testes associated with increased early-life nutrition have great potential to increase semen production potential, and therefore, net profitability of a bull. Typically, bulls are collected twice per day, two or three days each week in an AI setting (as long as they are producing good quality semen that is marketable). Consequently, these increases in marketable semen and profits would be substantial throughout the productive life of a bull, further emphasizing the potential for a net increase in profitability. The incremental cost of additional feed (based on actual amounts fed and current local feed prices) was approximately CAD$280 more for calves fed high- versus low-nutrition and only CAD$141 more for calves fed high- versus medium-nutrition. Although high-nutrition calves had a much higher level of feed intake, they were relatively small at the ages when the differential diets were fed, and as a consequence, incremental feed costs were modest (we assumed that costs associated with labor, bedding, housing, etc., were not affected). Even in the context of a traditional sire selection program, where bulls are collected at approximately 1 y of age and their semen used to produce offspring that will be subjected to rigorous testing, with the majority of bulls ultimately sent to slaughter, the cost of the extra feed early in life would be completely negligible compared to all the other costs of producing and maintaining these bulls for several years. Given the current practice of genomic selection, there is a trend in the industry to begin collecting bulls younger and younger, to be able to maximize use of their genetics as soon as possible. Therefore, bulls fed better early-life nutrition and
reached sexual maturity earlier would be of greater benefit as they could be younger when their semen is of marketable quality.

*In vitro* fertilization and assessment of embryo production is a well-established method to assess bull fertility (Larsson and Rodriguez-Martinez 2000), as pronuclear and cleavage rates in an IVF system were highly correlated with field fertility (Marquant-Le Guienne *et al.* 1990; Zhang *et al.* 1997). In this experiment, we used frozen semen from three bulls per nutrition group and three ejaculates from each of those bulls. Although the number of bulls per treatment group was limited, evaluating three IVF experiments per bull and using a relatively large number of oocytes was expected to accurately represent fertility of these bulls. There were no significant differences among groups in fertilizing ability or embryo production (at all stages evaluated). Although IVF has been reported to correlate with field fertility, it has also been suggested that evaluating sperm for more parameters than just IVF provides a better prediction of field fertility (Zhang *et al.* 1999; Kastelic and Thundathil 2008). Consequently, we also evaluated the proteome and viability of the frozen-thawed semen samples.

The use of 2D polyacrylamide gel electrophoresis is a well-established proteomics technique to identify proteins that are differentially expressed between samples (Hunt *et al.* 2005; Rahman *et al.* 2013). Proteomic studies of human and animal sperm have been widely reported, resulting in identification of numerous sperm-associated proteins and identification of many proteins involved in regulation of normal or defective sperm (Rahman *et al.* 2013; Holland and Ohlendieck 2015). Mature spermatozoa are transcriptionally and translationally inactive and therefore unable to synthesize proteins. Consequently, they are particularly interesting to study, as proteins present in sperm at the time of ejaculation will have a critical role in that cell’s ability to fertilize, and should provide insights regarding fertilizing ability of the bull. In the present
study, 2D gel analysis did not identify any protein spots differentially expressed among the three nutrition groups. Therefore, we concluded that the early-life nutritional modulation did not cause differences among groups in the sperm proteome. This was consistent with the conclusion that while early-life nutrition had profound effects on testes size (and to a lesser extent, age at puberty), it did not change sperm quality. In a previous study in dairy bulls, small differences between nutrition groups in sperm motility disappeared as bulls matured (Flipse and Almquist 1961). In the current study, detailed sperm assessments were only done at two time points (67 and 72 wk) and combined for proteomic analysis of these bulls. Therefore, in future studies, more frequent assessments starting soon after the onset of puberty would be warranted to determine whether nutrition affects semen quality soon after puberty.

There were no significant differences among nutrition groups regarding age at maturity, although bulls on high-nutrition diets were younger at puberty than low-nutrition bulls (Dance et al. 2015). Notwithstanding, there was a trend for the bulls in the high-nutrition group to be younger (∼ 20 d) at maturity than the bulls in the low-nutrition group. Similar trends were present among the groups with regards to the age at which semen samples were successfully chilled and cryopreserved; in that regard, bulls maintained on high-nutrition diets were younger (∼ 15 d) at these end points. Furthermore, bulls in the high-nutrition group appeared to have more rapid increases in the percentage of morphologically normal sperm than those in the other two groups (Figure 3-2). We speculated that if sample size was increased, these end points would have yielded a significant difference, consistent with the age at puberty results (Dance et al. 2015). Furthermore, the large variation in our age at maturity data were attributed to inconsistent reactions to the electroejaculator. Perhaps if an AV had been used, there would have been significant differences in this endpoint, similar to a recent report (Harstine et al. 2015).
Sperm viability assessed with SYBR14/PI was correlated with IVF results in bulls (Brito et al. 2003). Three populations of spermatozoa are identified with this method, green (viable), red (dead) and dual stained (moribund; Anzar et al. 2002; Grundler et al. 2004; Shojaei et al. 2012). The rate of transition of live sperm to moribund sperm has been implicated as a predictor of semen quality and the “proportion of moribund sperm has been shown to negatively correlate with fertility” (Shojaei et al. 2012). In the present study, there was a significant difference among nutritional groups in percentages of dead sperm, but not in percentages of live, moribund or moribund/total viable in the sperm viability parameters evaluated. It appeared that in high-nutrition bulls, some sperm died more quickly, resulting in a significantly higher percentage of dead sperm, and a somewhat lower percentage of moribund sperm (not significant), with “no significant difference” in live sperm. It was not clear why this occurred and further studies are indicated. Regardless, it is noteworthy that there were no significant differences among groups in the number of live sperm.

It is well established that nutritional modulation during gestation, early-life and post-puberty can have dramatic effects on reproductive potential of male mammals. Poor prenatal nutrition (due to inadequate maternal nutrition during gestation) reduced fertility and reproductive development of the offspring (Rae et al. 2002). In rams and rats (particularly males), inadequate nutrition during gestation delayed sexual maturity, decreased testicular volume and decreased plasma testosterone concentrations (Da Silva et al. 2001; Zambrano et al. 2005). In contrast, high prenatal nutrition increased Sertoli cell number in lambs (Alejandro et al. 2002). Furthermore, it is well established that high post-pubertal nutrition reduced daily sperm production, and increased the proportion of sperm abnormalities (Coulter and Kozub 1984;
Coulter et al. 1987; Coulter et al. 1997; Brito 2014), attributed, at least in part, to increased fat deposition in the scrotum (Brown 1994).

Our original intention was to confirm puberty in these bulls and subsequently move them to AI centre for semen collection (with an AV) and freezing. Unfortunately, this was not possible due to external circumstances. Consequently, semen collection for cryopreservation had to be done with an electroejaculator, making it very difficult to interpret the number of sperm collected. Therefore, we relied on literature values to estimate the number of harvestable sperm. Certainly, future studies, conducted at an AI centre and using an AV for semen collection, are needed to confirm the magnitude of the effect of early-life nutrition on the number of harvestable sperm. Regardless, the current study provided clear proof of concept that the differences are likely substantial, particularly with regard to the net economic benefits of increased early-life nutrition.

In conclusion, feeding bulls a higher level of nutrition from 2 to 31 wk would be beneficial to the AI industry. The resulting increase in profit from the additional harvestable semen outweighed the additional costs incurred by feeding the calves more from 2 to 31 wk of age. Furthermore, there were no indications that semen quality was significantly decreased in bulls fed a high level of nutrition early in life.

3.7 Acknowledgements

This work was supported by the Canadian Agriculture Adaptation Program (Ottawa, ON, Canada) and L'Alliance Boviteq Inc. (Saint-Hyacinthe, QC, Canada).
Chapter Four: **Higher early-life nutrition increases proliferation and maturation of Sertoli cells at 24 wk of age in Holstein bull calves**

4.1 Abstract

In Chapter 2, Holstein bull calves fed a high-nutrition diet (energy and protein) early in life were younger at puberty and had larger testes than calves fed a low-nutrition diet during the same interval. The objective of the current study was to evaluate how differential feeding affected testes.

In Holstein bull calves consuming a high-nutrition diet, we hypothesized that Sertoli cell proliferation begins earlier, leading to larger testes, and that Sertoli cells mature earlier, leading to an earlier onset of puberty compared to bull calves maintained on a low-nutrition diet. For this study, 32 Holstein bull calves were randomly allocated into two groups at approximately 1 wk of age to receive either a low- or high-nutrition diet from 2 to 32 wk of age. Three or four calves from each nutrition group were surgically castrated at 8, 16, 24, and 32 wk of age and testes were evaluated for expression of markers of immature, proliferating, and mature Sertoli cells.

Overall, we inferred that supplemental early-life nutrition increased proliferation and maturation of Sertoli cells; differences were apparent in many of the measured markers at 24 wk. Furthermore, we inferred that better early-life nutrition triggered more cellular proliferation in the testes, resulting in more Sertoli cells, thereby leading to larger testes.

4.2 Introduction

We reported that calves fed a high-nutrition diet (energy and protein) early in life attained puberty earlier and had larger testes than calves fed a low-nutrition diet during the same interval (Dance *et al.* 2015). Furthermore, these differences were maintained post-puberty, resulting in
adult bulls with larger testes, although semen quality was not significantly affected (Dance et al. 2016). In the artificial insemination industry, bulls with larger testes have greater sperm production potential, which is a potential economic benefit. However, molecular mechanisms causing larger testis in bulls maintained on high-nutrition diets remain unknown.

Reproductive development of the bull can be divided into three periods: infantile, prepubertal, and pubertal. The infantile period comprises 0 to 8 wk of age and is characterized by low secretions of both gonadotropins and testosterone. The prepubertal period (8 to 20 wk) is characterized by transient increases in gonadotropin secretion (particularly LH; so called “early gonadotropin rise”) and a concurrent increase in testosterone secretion (Barth et al. 2008). In the post-pubertal bull, every GnRH pulse is associated with pulsatile secretion of gonadotropins and testosterone. Approximately 4 to 8 pulses occur per day in adult bulls (Senger 2003). Prior to 25 wk in bull calves, testes are comprised of pre-spermatogonia, spermatogonia, adult Leydig cells, and undifferentiated Sertoli cells. During this stage, testicular growth occurs slowly. Thereafter (pubertal period), there is rapid testicular development through to puberty. Histological changes in the testes include a marked increase in diameter and length of seminiferous tubules, proliferation and differentiation of germ cells, and development of adult Leydig cells (30 wk), Sertoli cells (30 to 40 wk), and mature sperm (32 to 40 wk; Rawlings et al. 2008).

There are two discrete Sertoli cell populations, referred to as fetal and adult; those populations differ in morphology, function, and age at which they appear in the testis. During the prepubertal period, Sertoli cells undergo terminal differentiation and maturation to form adult cells, the population of Sertoli cells present in post-pubertal testes. The change from a fetal to adult Sertoli cell population is characterized by morphological and functional changes, loss of proliferative ability, and formation of inter-Sertoli cell tight junctions (Tarulli et al. 2012). Only
immature Sertoli cells can proliferate; therefore, the post-pubertal Sertoli cell number is determined before puberty. Markers for immature Sertoli cells include anti-mullerian hormone (AMH), cytochrome P450 (P450) and neural cell adhesion molecule (NCAM) whereas AR and P27 are markers for mature Sertoli cells. Since efficiency of spermatogenesis is influenced by number of Sertoli cells, factors affecting Sertoli cell proliferation during the prepubertal period ultimately influences testis size and daily sperm production in post-pubertal bulls (Sharpe et al. 2003).

In our previous study, daily sperm production (DSP) per gram of tissue was not affected by diet (Dance et al. 2015). We inferred that early-life nutritional modulation did not affect the efficiency of spermatogenesis, or alter the capacity of the Sertoli cells to support developing germ cells. Improved early-life nutrition, however, caused bulls to have larger testes, which increased sperm production.

The objectives of this study were to evaluate testicular expression of IGF-I and IGF-IR in low- versus high-nutrition bulls and to evaluate proliferation and differentiation of Sertoli cell precursors. In Holstein bull calves consuming a high-nutrition diet, we hypothesized that Sertoli cell proliferation begins earlier, leading to larger testes, and that Sertoli cells mature earlier, leading to an earlier onset of puberty, compared to bull calves maintained on a low-nutrition diet.

4.3 Materials and methods

4.3.1 Bulls and treatments

Thirty-two Holstein bull calves were randomly allocated into two nutritional groups at approximately 1 wk of age to receive either a low-, or high-nutrition diet from 2 to 32 wk of age. Calves were fed 4 or 8 L/day (low and high diets respectively) of reconstituted milk replacer
from 2 to 8 wk of age, as described (Dance et al. 2015). Calves were then transitioned onto a barley silage-based diet (forage source for all diets) and fed a low- or high-nutrition diet from 8 to 32 wk. Diets composition are shown in Table 4-1. The low-nutrition diet had 12% CP, 64.4% TDN (CP and TDN are reported on a DM basis) and the high-nutrition diet had 20% CP and 71.6% TDN. These diets were prepared and classified as low and high levels of nutrition, based on diets used in our previous study (Dance et al. 2015). Feed intake was also controlled as described (Dance et al. 2015). Once a month, body weight was measured using a weight tape. This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley silage</td>
<td>98.5</td>
<td>55.5</td>
</tr>
<tr>
<td>Rolled barley</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>Canola meal</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td>Mineral/Vit Mix</td>
<td>1.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

4.3.2 Castrations

Three or four calves from each nutrition group were surgically castrated at 8, 16, 24, or 32 wk of age. Castrations were done under caudal epidural anesthesia (0.07 mg/kg BW of xilazine, in 3 mL saline) and calves were given meloxicam (0.5 mg/kg BW). Testes were recovered and weighed. Testicular parenchyma was cut into cubes (approximately 10 mm in
each dimension) and snap-frozen on dry ice for subsequent PCR and protein work. This work was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee and was conducted in accordance with the standards of the Canadian Council on Animal Care.

4.3.3 Tubular diameter

Recovered testicular tissues were processed for histology, as described (Chapter 2). Testes were cut longitudinally and 4 x 4 x 4 mm pieces of tissues were fixed in modified Davidson’s fixative for 24 h. Thereafter, the fixative was removed and the samples were washed in 70% ETOH three times at 24 h intervals, and then samples were embedded in paraffin blocks, sectioned (4 µm) and stained with hematoxylin and eosin. Twenty round tubules from each bull were imaged (100X objective, Leica microsystems GmbH Wetzlar, Germany) and their diameters measured (Image J, National Institutes of Health, Bethesda, MD, USA).

4.3.4 Testicular gene expression

4.3.4.1 Isolation of RNA.

Total cellular RNA was isolated as described in Chapter 3.

4.3.4.2 Real-Time RT-PCR

Real-Time RT-PCR was conducted as described in Chapter 3. Testicular samples were evaluated for expression of AMH, AR, IGF-I, IGF-IR, Ki67, NCAM, P27, P450 and proliferating cell nuclear antigen (PCNA). Each PCR was performed in duplicate with non-reverse transcription and no cDNA controls included. Mean threshold cycle (Cq) was determined for
each sample to evaluate changes in gene expression. The C_q was normalized to the reference gene \textit{GAPDH}, as described (Klein \textit{et al.} 2010), producing delta (\(\Delta\)) C_q values. The PCR results were displayed as \(1/\Delta C_q\), to reflect actual expression patterns of genes.

### Table 4-2. Primer sequences used to characterize gene expression in testicular tissues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession #</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>NM_173890.1</td>
<td>TGGAAATGGTGCGCTCCTG</td>
<td>GGTCTTTCTGTAAGGCACAGC</td>
<td>82</td>
</tr>
<tr>
<td>AR</td>
<td>NM_001244127.1</td>
<td>GCCCTGACCTGGTTTCCA</td>
<td>CATTCGGACACACTGGCTGTA</td>
<td>69</td>
</tr>
<tr>
<td>IGF-I</td>
<td>NM_001077828.1</td>
<td>GGTGAAGATGCCATCACATC</td>
<td>GCTGGTAAGGCGAGCA</td>
<td>91</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>XM_010816884.1</td>
<td>TCAAGAGTTATCTCGTCTCTGA</td>
<td>CCAGCCATCTGGATCATTTTG</td>
<td>91</td>
</tr>
<tr>
<td>Ki67</td>
<td>XM_002698582.3</td>
<td>AAGAGGAGAGACGCAAGC</td>
<td>GGGGCTGCTCCTTGATGATT</td>
<td>83</td>
</tr>
<tr>
<td>NCAM</td>
<td>XM_010812357.1</td>
<td>GCCGTGATCGTGTGTGTATG</td>
<td>GCCCTTTGTGCTTCCAGATG</td>
<td>64</td>
</tr>
<tr>
<td>P27</td>
<td>NM_001100346.1</td>
<td>ACACGCATTTGGTGAATG</td>
<td>AGGCCACTGCTCCGCTAAC</td>
<td>68</td>
</tr>
<tr>
<td>P450</td>
<td>AY265992.1</td>
<td>GCTGCATGAGATCCAGAGAC</td>
<td>GCCAGCAATAGCTAAGACCCA</td>
<td>74</td>
</tr>
<tr>
<td>PCNA</td>
<td>NM_001034494.1</td>
<td>GAACCTCACAGCATGCTCA</td>
<td>ACGTCCGCGTTATCCTCA</td>
<td>86</td>
</tr>
</tbody>
</table>

### 4.3.5 Immunohistochemistry

For PCNA immunohistochemistry, 4 \(\mu\)m sections were cut from the same tissue blocks used to assess tubular diameter and placed on charged slides. Sections were deparaffinized in xylene and rehydrated through a decreasing alcohol gradient. Antigen retrieval was performed.
for 10 min in 10 mM sodium citrate buffer (pH 6.0) on a hot plate at 95 °C (to ensure even heating). Sections were then cooled to room temperature, washed in PBS, and subsequently blocked in CAS block (Invitrogen) for 10 min at room temperature. Sections were exposed to primary antibody developed against a proliferation marker (PCNA, 1:300, M0879 DAKO, Mississauga, ON, Canada) overnight at 4 °C, and the negative control was incubated with CAS block overnight. The following morning, cells were washed three times with PBS (5 min each) and then incubated with secondary antibody (1:500, Donkey-anti mouse Alexa 555, A31579, Invitrogen). Cells were washed three times (5 min each) with PBS, then mounted with VectaShield mounting medium with DAPI (H-100, Vector Laboratories, Burlingame, CA, USA) and imaged on a Leica DFC 300FX microscope.

4.3.6 Statistical analysis

Statistical analyses were conducted using SPSS Version 22 (IBM SPSS Statistics for Macintosh, Version 22.0., IBM Corp., Armonk, NY, USA). A multivariate ANOVA response model was used to determine and locate effects of nutrition (low and high), age, and the nutrition x age interaction, on BW, testes weight, tubular diameter and all gene expression data. If either the main effect or the interaction was significant (Wilks Lambda), differences were located using Tukey’s HSD tests. If data were not normally distributed, a Mann-Whitney U test for independent samples was used. Data is reported as means (± SEM).
4.4 Results

4.4.1 Body weight and testes size

Body weight of the bulls was similar to those in our previous study, with high-nutrition bulls being larger than low-nutrition bulls throughout the experimental period (Figure 4-1). Bull calves on the high-nutrition diet had a trend towards having larger testes than those on the low-nutrition diet throughout the experiment. At 24 wk, high-nutrition bulls had larger testes (P < 0.05) than the low-nutrition group (Figure 4-2).

Figure 4-1. Mean (± SEM) BW of Holstein bulls fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.

*Difference (P < 0.05) between groups within an age.
Figure 4-2. Mean (± SEM) testes weight of Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.

A,BWithin an age, difference (P < 0.05) between diets.

4.4.2 Tubular diameter

Representative images of tubular diameter of each nutrition group at each age are shown in Figure 4-3. There were effects of age (P < 0.001) and a tendency for a nutrition x age interaction (P = 0.055) on testes tubular diameter. Bulls fed the high-nutrition diet had larger tubular diameter at 24 wk (P < 0.05) than those fed a low-nutrition diet. High-nutrition bulls had a trend towards having a larger tubular diameter at 32 wk (Figure 4-4).
Figure 4-3. Representative images of tubular diameter of the seminiferous tubules of Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk and castrated at four ages.
Figure 4-4. Mean (± SEM) tubular diameter of Holstein bulls fed low- or high-nutrition diet (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.

A,B Within an age, difference (P < 0.05) between diets.

4.4.3 Mature Sertoli cell markers

The expression patterns of two known markers of mature Sertoli cells (AR and P27) were evaluated in these bull calves. Expression of both markers was higher (P < 0.05) in high- versus low-nutrition calves at 24 wk (Figure 4-5). However, at no other time points did the nutrition groups differ significantly.

4.4.4 Immature Sertoli cell markers

Expression of three markers for immature Sertoli cells (AMH, P450, and NCAM) was evaluated. Expression of AMH decreased from 8 to 32 wk. There was effects of age (P < 0.036) however there was no treatment effects (P = 0.617) and no nutrition x age interaction (P = 0.991). At 8 wk, AMH expression was different (P = 0.018) from all other age groups and
independent of nutritional group (Figure 4-6A). For \textit{P450} and \textit{NCAM}, there was no effects of age nor treatment (Figure 4-6 B and C).

\textbf{4.4.5 Proliferation markers}

We evaluated the expression of two cell proliferation markers, \textit{Ki67} and \textit{PCNA}).

Expression of both markers was higher (P < 0.05) in high- versus low-nutrition calves at 24 wk (Figure 4-7). For \textit{Ki67}, regardless of nutrition group, there was an age effect between the 8 and 16 wk groups (P = 0.001) and the 16 and 32 wk groups (P = 0.011; Figure 4-7A). \textit{PCNA} expression within each nutrition group had significant differences among ages. For the low-nutrition group, the 32 wk group significantly differed in comparison to all other age groups, whereas for high-nutrition groups, both the 8 and 16 wk-old groups differed from the 32 wk group (Figure 4-7B). Based on immunohistochemistry, \textit{PCNA} was expressed in Sertoli cells of 24 wk-old calves (Figure 4-8). A representative image of the PCNA negative slide is show (Figure 4-9).
Figure 4-5. Mean (± SEM) expression (1/ ΔCq) of A) AR and B) P27 in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.

A, B Within an age, difference (P < 0.05) between diets.
Figure 4-6. Mean (± SEM) expression ($1/ΔC_q$) of A) AMH, B) P450 and C) NCAM in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.
Figure 4-7. Mean (± SEM) expression (1/ΔCq) of A) *PCNA* and B) *Ki67* in Holstein bulls fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk of age and castrated at four ages.

Within an age, diets without a common superscript differed (P < 0.05).
Figure 4-8. Representative images of IHC of seminiferous tubules of 24 wk-old calves immunolabelled for PCNA.

PCNA (right panel) expression in seminiferous tubules counter-stained with DAPI (nuclear marker; left panel).

Figure 4-9. Representative negative image of IHC of seminiferous tubules of 24 wk-old calves immunolabelled for PCNA and counter stained with DAPI.

Negative control for PCNA (right panel) expression in seminiferous tubules counter-stained with DAPI (nuclear marker; left panel).
4.4.6 IGF-I markers

Expression patterns of IGF-I was higher (P < 0.05) in high- versus low-nutrition calves at 24 wk of age (Figure 4-10A). However, neither diet nor age affected expression of IGF-IR (Figure 4-10B).

Figure 4-10. Mean (± SEM) expression (1/ ∆Cq) of A) IGF-I and B) IGF-IR in Holstein bull calves fed low- or high-nutrition diets (n = 3 or 4 bulls/diet/age) from 2 to 32 wk and castrated at four ages.

A,BWithin an age, diets without a common superscript differed (P < 0.05).
4.5 Discussion

In our previous experiment (Chapter 2), high-nutrition diets (both energy and protein) during early-life (2 to 31 wk) produced bulls that were younger at puberty and had larger testes than bulls fed a low-nutrition diet. We duplicated the previous nutritional modulation in the present study to evaluate effects of early-life nutrition on prepubertal testes. Hormone concentrations, SC and PTV were not assessed, as we have already determined effects of early-life nutrition on these characteristics. Consistent with our previous data, bull calves on the high-nutrition diet were larger (BW) and had larger testes than those calves fed a low-nutrition diet. That testes were significantly larger only at 24 wk was attributed to sample size or variation within groups, as the high-nutrition group had a trend for larger testes throughout the entire experimental period. Notwithstanding, that testis weight significantly differed between nutrition groups at 24 wk indicated that nutrition had a critical role during this period of testicular development.

High-nutrition bulls had a larger average seminiferous tubular diameter than their low-nutrition counterparts at 24 wk. Seminiferous tubular diameter is associated with testes maturation and increases five-fold from birth to adulthood (Abdel-Raouf 1960; Curtis and Amann 1981; Evans et al. 1996; Brito 2006). Curtis and Amann 1981 reported that rapid testicular growth occurs between 24 and 32 wk, consistent with this study. These authors attributed this increase in testis weight to an increase in seminiferous tubular diameter, total tubule length, and an “increase in the proportion of the parenchyma occupied by seminiferous tubules” (Curtis and Amann 1981). Additionally, they suggested that the proportion of testicular parenchyma occupied by the seminiferous tubules in 32 wk-old bulls was comparable to that of mature bulls. They suggested that further growth of the testes after 32 wk was due to an increase
in total tubule length and to a lesser extent, an increase in tubular diameter (Curtis and Amann 1981). Comparing the present data to our previous data from adult bulls (Dance et al. 2015), we would have expected an increase in tubular diameter from 32 wk to adulthood. However, it is important to note that tubular diameter of the adult bulls was not different between nutritional groups, suggesting that total tubule length must have been longer to account for larger testes in high-nutrition bulls. In the present study, the high-nutrition bulls must be reaching mature tubular diameter earlier and therefore start tubular elongation earlier, with both of these resulting in larger testes.

Sertoli cells are a major somatic cell of the testes, with a function to support germ cells during spermatogenesis. There is a fetal population of Sertoli cells present from birth that proliferate and subsequently differentiate into the adult Sertoli cell population (Tarulli et al. 2012). However, like most cell populations, it is not uniform, as some cells are ceasing proliferation and beginning differentiation whereas others are still proliferating. This creates a period where these cell types have substantial overlap and occur simultaneously. In the present study, there was increased expression of markers indicating maturation and proliferation of Sertoli cells in high- versus low-nutrition calves, particularly at 24 wk.

Expression of three known markers of immature Sertoli cells (AMH, P450, and NCAM) were assessed. Anti-mullerian hormone (AMH) is responsible for regression of the Mullerian ducts very early on in fetal development and therefore plays a critical role in the male sex determination. The AMH expression begins very early in fetal Sertoli cells and maintains a high level of expression during Sertoli cell maturation, although it is down-regulated around puberty, coinciding with final maturation of the Sertoli cell population (Behringer et al. 1994; Sharpe et al. 2003). As bulls matured, AMH expression decreased, as expected. However, there were no
differences in *AMH* expression between low- and high-nutrition groups. Therefore, we concluded that there were similar numbers of immature Sertoli cells in bull calves in each treatment group, regardless of age. *NCAM* is expressed by fetal Sertoli cells. It is believed to have a role during gonocyte migration to the basement membrane of the seminiferous tubule and is down-regulated during maturation (Orth and Jester 1995; Sharpe *et al.* 2003). In this study, *NCAM* expression patterns were similar to *AMH*, with a general trend of decreasing expression throughout the experimental period. Although expression of *P450* (also known as aromatase, a steroidogenic enzyme) is high in fetal Sertoli cells, it is down-regulated during maturation. In adult testes, *P450* expression mainly occurs in Leydig cells. Since total RNA extracted from the testes was used, perhaps these results were confounded by contributions of adult Leydig cells to expression of *P450*.

Androgen receptor (AR) is a well-established marker of mature Sertoli cells. Expression of this marker increases prior to final maturation of the Sertoli cells, indicating it has a role in maturation (Sharpe *et al.* 2003). It is noteworthy that absent or low expression of *AR* can lead to failure of maturation of Sertoli cells in humans, based on nuclear and general morphology (Regadera *et al.* 2001). In the current study, there was a trend for both nutrition groups to have increasing levels of *AR* with increasing age, consistent with *AR* being a marker of Sertoli cell maturation. Notwithstanding, it was noteworthy that the high-nutrition group had increased expression of *AR* at 24 wk compared to the low-nutrition group; therefore, we inferred that testes of high-nutrition bulls were more mature than those of the low-nutrition group at 24 wk. This was consistent with our previous report that a high-nutrition diet during early-life caused bulls to mature faster and to be younger at puberty than those fed a low-nutrition diet during early-life (Dance *et al.* 2015). In addition, *P27* is another known marker associated with Sertoli cell
maturation; it is a cyclin-dependent kinase inhibitor expressed with inhibition of proliferation 
(Beumer et al. 1999). Expression of P27 was significantly higher in the high-nutrition calves 
compared to the low-nutrition bulls at 24 wk; consistent with AR.

*PCNA* and *Ki67* are both cell cycle-associated proteins, necessary for proper DNA 
replication, and are known markers for proliferation of Sertoli cells (Iatropoulos and Williams 
1996). In the present study, both of these proliferation markers were expressed at a higher level 
in high- versus low-nutrition calves at 24 wk. This trend continued until 31 wk (although with no 
significance). Therefore, we inferred that high-nutrition calves had more proliferating Sertoli 
cells at this age. Since PCR was done on total testicular protein, it cannot be definitively 
determined if both *Ki67* and *PCNA* were expressed exclusively by Sertoli cells in this study. 
Regardless, based on IHC for *PCNA*, it was clear that Sertoli cells had the highest expression of 
*PCNA* at 24 wk. This supported the conclusion that Sertoli cells were proliferating at a higher 
rate in the high-nutrition calves at this age.

Overall, it appeared that nutritional modulation was increasing proliferation as well as 
maturation of Sertoli cells in high- versus low-nutrition calves. This effect of shifting some of the 
developmental timelines to younger ages in high-nutrition calves was readily apparent by 24 wk. 
Consequently, the following question arises: Why is the diet causing a shift? This could be 
attributed to IGF-I (Chapter 2), where we detected dramatic differences in IGF-I concentrations 
during the differential feeding period. In rodents, IGF-I is known to enhance proliferation of 
Sertoli cells. It has been shown that FSH, one of the key mitogenic factors of Sertoli cells, cannot 
exert its effect on Sertoli cells without the IGF-I signaling pathways. In the present study, there 
was also a difference in IGF-I expression between low- and high-nutrition calves at 24 wk,
reinforcing the hypothesis that IGF-I has a role in relaying nutritional status of the body to the testes.

In this study, 24 wk seemed to be a critical period of development in bull calves, with significant differences between the two diets at this age. However, it is important to note that we investigated these calves at relatively wide intervals (8, 16, 24, and 32 wk); it would be interesting to further investigate the 16-24 wk age in more detail to determine when these differences are manifesting. For this reason, we cannot definitively say that 24 wk is the time when the testicular differences between high- and low-nutrition calves are beginning. Mature Sertoli cell markers were expressed at higher levels in high-nutrition calves at 24 wk and there was also more proliferative cells in their testes at this age. Therefore, we inferred that better early-life nutrition triggered more cellular proliferation in the testes, resulting in more Sertoli cells. Additionally, perhaps 24 wk could be used as a time to evaluate bulls for hastened or delayed maturation, depending on their testes size at this age, as we know that the differences are manifested by this age.

This data in combination with previous reports prompted me to further investigate the role of IGF-I in Sertoli cell development (Chapter 5).

4.6 Acknowledgments

I would like to thank Gerrit Van Hierden for supplying the calves and allowing us to work on their farm. Additionally, I would like to thank William Torres and the rest of the research team at Cattleland Feedyards Ltd. for all their help throughout the study.
Chapter Five: **A combination of IGF-I and FSH promotes proliferation of prepubertal bovine Sertoli cells isolated and cultured in vitro**

5.1 Preamble

The majority of the study described in this chapter has been submitted to *Reproduction, Fertility and Development*.

Title: A combination of IGF-I and FSH promotes proliferation of prepubertal bovine Sertoli cells isolated and cultured *in vitro*

A. Dance, J. Kastelic and J. Thundathil

A.D performed 100% of the experiments, analyzed 100% of the results and contributed 95% of the writing, J.K and J.T both supervised the project and reviewed the manuscript.

A preliminary study using cells isolated from 8, 16, 24 and 32 wk-old bull claves enabled us to optimize the materials and methods for this experiment. The results of these preliminary studies are shown in Appendix B.

5.2 Abstract

Beef and dairy bull calves fed a low-nutrition diet during early-life had decreased concentrations of circulating IGF-I, delayed increase in testosterone, smaller testes and delayed puberty compared to those fed high-nutrition diets. Although IGF-I has important roles in Sertoli cell function in rats and mice, this has not been well documented in bulls. Objectives were to: 1) isolate Sertoli cells from bull calves at 8 wk of age; 2) culture them *in vitro*; and 3) determine effects of IGF-I, FSH, and a combination of both hormones, on cell proliferation. For Sertoli cell
isolation, minced testicular tissues were treated with collagenase followed by trypsin and
hyaluronidase to digest seminiferous tubules and release Sertoli cells.

In this study, Sertoli cells were successfully isolated from 8 wk-old Holstein bull calves
(n = 4) and these cells were cultured for up to 8 d. A combination of IGF-I and FSH increased
proliferation (~18%) and therefore cell number (1.5 fold) of prepubertal bovine Sertoli cells in
culture, providing clear evidence that IGF-I has a similar role in bovine Sertoli cells as reported
in rodents.

**Keywords:** Cell proliferation, cell culture, cell isolation, testicular somatic cells.

### 5.3 Introduction

In beef and dairy bull calves fed a low-nutrition diet early in life (before ~30 wk), there
were decreased concentrations of circulating IGF-I, delayed increases in systemic testosterone
concentrations, smaller testes and delayed puberty compared to bulls fed high-nutrition diets
(Brito *et al.* 2007a; Dance *et al.* 2015). This highlighted a potential role for circulating IGF-I in
hastening testes maturation and therefore puberty in bulls. However, more investigation is
needed to determine if IGF-I affects proliferation of testicular somatic cells, in particular, Sertoli
cells.

Sertoli cells have numerous functions, including providing developing germ cells with
nutrients and structural support (Chang *et al.* 2011), coordinating spermatogenesis, and forming
the blood testes barrier that allows developing germ cells to mature in an immune-privileged
environment. There are two discrete Sertoli cell populations (fetal and adult) that differ in
morphology, function, and age at which they appear in the testis (Sharpe *et al.* 2003). The change
from fetal to adult populations of Sertoli cells is characterized by loss of proliferative ability, and formation of inter-Sertoli cell tight junctions (Tarulli et al. 2012). Only immature Sertoli cells can proliferate; therefore, post-pubertal Sertoli cell number is determined before puberty. Adult Sertoli cells have a finite number of germ cells that they can support (Orth et al. 1988; Sharpe 1994). Consequently, efficiency of spermatogenesis is influenced by Sertoli cell number, and factors affecting Sertoli cell proliferation during the prepubertal period influence testis size and daily sperm production (Sharpe et al. 2003).

Insulin-like growth factor-I (IGF-I) has received considerable attention regarding its roles in reproduction and testicular development (Baker et al. 1996; Griffeth et al. 2014). Receptors for IGF-I (IGF-IR) are present in GnRH neurons, with increasing numbers of these receptors during sexual development (Daftary and Gore 2004). In addition, synthesis of IGF-I has been demonstrated in somatic cells within the testis (Cailleau et al. 1990; Wang and Hardy 2004) and the presence of both IGF-I and IGF-IR have been demonstrated in Sertoli cells, Leydig cells, and spermatocytes of various species (Hansson et al. 1989; Wang and Hardy 2004; Villalpando et al. 2008), suggesting an important autocrine and/or paracrine function of this hormone in these cells. It is noteworthy that IGF-I increases Sertoli cell proliferation during embryonic days 14 to 18 in the mouse (Villalpando et al. 2008). In contrast, in knockout mice lacking IGF-IR in Sertoli cells, there was increased Sertoli cell death and decreased Sertoli cell proliferation, which reduced the number of viable Sertoli cells and highlighted the importance of IGF-I in regulation of Sertoli cell function (Froment et al. 2007). Furthermore, in immature Sertoli cells, insulin/IGF signaling pathways are required for FSH to mediate its proliferative effects (Pitetti et al. 2013).

Since Sertoli cells have a critical role in spermatogenesis, it is important to isolate and study these cells to understand their function. However, much of the work regarding Sertoli cell
biology has been done in laboratory rodents, with limited studies on cattle. Bulls are born with fetal Sertoli cells that ultimately undergo terminal differentiation and maturation to form an adult Sertoli cell population, which is in place by 30 to 40 wk (Rawlings et al. 2008). There are very few publications regarding isolation of Sertoli cells from bovine testes, with apparently none done at 8 wk of age. Additionally, our previous work demonstrated larger testes, earlier puberty and increased IGF-I concentrations in high-nutrition bull calves, which led to the hypothesis that increased IGF-I concentrations in Holstein bulls promote Sertoli cell proliferation and increase testes size (Dance et al. 2015). Therefore, objectives were to: 1) isolate Sertoli cells from bull calves at 8 wk of age; 2) culture them in vitro; and 3) determine effects of IGF-I, FSH, and a combination of both hormones, on cell proliferation.

5.4 Materials and methods

5.4.1 Bulls

Holstein bull calves (n = 4) were castrated at 8 wk of age. Intact testes were placed in sterile phosphate buffered saline (PBS) with gentamicin (50 µg/mL, G1397, Sigma, St. Louis, MO, USA) and transported to the laboratory on ice. Castrations were done under caudal epidural anesthesia (0.07 mg/kg BW of xylazine, in 3 mL saline) and calves were given meloxicam (0.5 mg/kg BW). This work was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee and was conducted in accordance with the standards of the Canadian Council on Animal Care.
5.4.2 Cell isolation

Sertoli cells were isolated using a procedure modified from previous reports (Anway et al. 2003; Oliveira et al. 2009; Chang et al. 2011) with the following modifications. Upon arrival at the laboratory, intact testes (Figure 5-1A) were washed in Hanks balanced salt solution (HBSS, 14175-095, Life Technology, Burlington, ON, Canada). Testes were de-capsulated (Figure 5-1B) and washed in HBSS with 50 µg/mL gentamicin. Approximately 5-10 g of tissue per bull were extensively minced (with scissors) into very fine pieces (1-4 mm; Figure 5-1C) and placed in a 250 mL Erlenmeyer flask with 50 mL enzyme solution 1 (DMEM/F12; (11320-033, Life Technology), 1 mg/mL collagenase (C5138, Sigma, St. Louis, MO, USA), and 50 µg/mL DNase; DN25, Sigma)) for 30 min at 35 °C in a shaking water bath (110 oscillations/min). After incubation, tubules were pelleted by centrifugation (300 x g for 3 min). The supernatant was discarded and tubules were washed once with DMEM/F12 by centrifugation at 300 x g for 3 min. Tubules were then treated with 1 M glycine (G8898, Sigma) and 2 mM EDTA (E5134, Sigma), in PBS (pH 7.4) containing 50 µg/mL DNase at room temperature for 10 min, with gentle pipetting during incubation to lyse Leydig cells. Then, tubules were isolated by centrifugation (300 x g, 3 min), supernatant removed and the resulting pellet washed three more times (300 x g, 3 min) with DMEM/F12.
Figure 5-1. Bovine testes during Sertoli cell isolation procedure.
A) intact testes; B) de-capsulated testes; and C) minced testes.

Tubules were then incubated in enzyme solution 2 (50 mL DMEM/F12 containing 1 mg/mL trypsin; T8003, Sigma and 50 µg/mL DNase) for 30 min at 35 °C in a shaking water bath (130 oscillations/min). At this stage, tubules were partly digested, with germ cells and Sertoli cells being released (Figure 5-2). After incubation, 0.5 mg/mL trypsin inhibitor STI (T9128, Sigma) was added to enzyme solution 2 to stop trypsin action. Digested tubules were pelleted by centrifugation (800 x g for 3 min). The resulting pellet was re-suspended in DMEM/F12 containing 0.5 mg/mL bovine serum albumin (BSA, A7030, Sigma) to prevent cell aggregation. Thereafter, cells were washed by centrifugation (800 x g for 3 min) and incubated in enzyme solution 3 (50 mL DMEM/F12 supplemented with 1 mg/mL collagenase, 1 mg/mL hyaluronidase (H3506, Sigma), 0.1 mg/mL STI, and 50 ug/mL DNase) for 30 min at 35 °C in a shaking water bath (100 oscillations/min). After this incubation, tubules were dispersed by gentle pipetting for 5-10 min. At this point, germ cells and Sertoli cells should be separated from the tubule wall (Figure 5-3). Following gentle pipetting, the cell suspension was centrifuged (800 x g for 3 min), the supernatant discarded, and tubules were washed three times with DMEM/F12 containing 0.05% BSA (centrifugation at 800 x g for 3 min each). After the final washing, the
cell pellet was re-suspended in 50 mL DMEM/F12 containing 0.05% BSA and passed through a 100-µm cell strainer (BD Falcon), which retained intact tubules, but allowed passage of Sertoli and germ cells (Figure 5-4). Cells in the filtrate were washed three times with DMEM/F12 (centrifugation at 800 x g for 3 min). Following the final wash, cells were re-suspended in 20 mL of Sertoli cell medium (DMEM/F12 supplemented with (all products from Sigma) 5 µg/mL human transferrin (T8158), 2.5 ng/mL Epidermal growth factor (EGF; E9644), 20 µg/mL gentamycin, 10 µg/mL bacitracin (B0125), and 6.7 ng/mL selenium (S5261).

Figure 5-2. Bovine seminiferous tubules (40X) after enzyme digestion 2.
Enzyme digestion 2 = 50 mL DMEM/F12 containing 1 mg/mL trypsin and 50 µg/mL DNase.
Figure 5-3. Bovine seminiferous tubules (40X and 100X) after enzyme digestion 3.
Enzyme digestion 3 = 50 mL DMEM/F12 supplemented with 1 mg/mL collagenase, 1 mg/mL hyaluronidase, 0.1 mg/mL STI and 50 ug/mL DNase.

Figure 5-4. Cells after passage through cell strainer (200X).
The cell strainer allowed Sertoli and germ cells to pass through, but retained intact tubules, thereby resulting in a cell suspension free of tubules.
5.4.3 Sertoli cell number and viability

Prior to cell plating, Sertoli cell number was determined using a hemocytometer. Following the Sertoli cell isolation procedure, trypan blue (0.4%) was used to determine cell viability. Cells were plated (2 mL at 0.6 x 10^6 cells/mL) in 35-mm cell culture plates. Day of isolation was considered Day 0, media was changed 24 h after plating (to remove floating germ cells), with Sertoli cells attached to the bottom of the plate by 24 h after the onset of culture. Thereafter, media was changed every 48 h. Phase images of cells were viewed with an Olympus CKX41 microscope and recorded with an Infinity 3 camera.

5.4.4 Analyses of purity of Sertoli cells by immunofluorescence microscopy

Purity of Sertoli cell cultures was evaluated by immunofluorescence on Day 5 of culture. Cells were washed once with PBS to remove media and then fixed with 4% PFA (P6148, Sigma) for 15 min at room temperature. Cells were then washed with 0.1% Triton X-100 (T8787, Sigma) in PBS for 15 min, washed twice with PBS (5 min each), and blocked with CAS block (diluted 1:1 with PBS; Invitrogen) for 15 min at room temperature. Cells were exposed to primary antibody developed against a Sertoli cell marker (GATA4, 1:50 C-20 Santa Cruz) or a Leydig cell marker (3β-HSD, 1:50 P-18 Santa Cruz) overnight at 4 °C. No primary antibody was added to the secondary antibody controls. Additionally, a negative control with Bovine aortic endothelial cells (BAE) was included, which should not express GATA4 or 3β-HSD. The following morning, cells were washed three times with PBS (5 min each) and then incubated with secondary antibody (1:500, donkey-anti goat Alexa 488, A11055, Invitrogen). Cells were washed three times (5 min each) with PBS then mounted with vectashield mounting medium
with DAPI (H-100, Vector Laboratories, Burlingame, CA, USA) and imaged on a Leica DFC 300FX microscope. A minimum of 100 cells were counted per bull to determine culture purity.

5.4.5 Protein extraction and western blotting

Total protein extracts were prepared from Sertoli cells on Day 5 to confirm immunofluorescence results. Protein extraction was done on ice with RIPA buffer (R0278, Sigma) for 1 h; samples were vortexed every 15 min, and at the end of the extraction period, samples were centrifuged (12000 x g, 5 min) and supernatant frozen. Western blotting was done as described (Shojaei Saadi et al. 2013), using the same antibodies as for immunofluorescence (1:200). Total cell protein was quantified using the RC DC protein assay (Bio-Rad).

5.4.6 Hormone treatments

On Day 7 of culture, cells were treated with recombinant human IGF-I (291-G1, R&D Systems) at 20 mg/mL (IGF-I 20) or 300 ng/mL (IGF-I 300), FSH (bovine FSH, Sioux Pharmaceuticals; 0.4 ng/mL), IGF-I and FSH together at 20 ng/mL IGF-I and 0.4 ng/ml FSH (IGF-I 20 + FSH) or 300 ng/mL IGF-I and 0.4 ng/mL FSH (IGF-I 300 + FSH), or no exogenous hormones (control). Human IGF-I is 95% homologous with bovine 1GF-I. Day 7 was chosen based on previous Sertoli cell work (Khan et al. 2002; Froment et al. 2007) and hormone concentrations were based on a previous study evaluating in vivo IGF-I concentrations in Holstein bulls (Dance et al. 2015). After 24 h of exposure to hormones, cells were evaluated for cell number and intensity of 5-ethynyl-2’–deoxyuridine (EdU) reaction to signify proliferation.
5.4.7 Evaluating cell number

Cells were removed from the culture dishes using acutase (561527, BD Biosciences) for 10 min at 37 °C. Cells were collected, and the plates washed and checked to ensure all cells had been removed. The cells were then centrifuged (800 x g, 3 min). Supernatant was discarded and cells were washed twice in DMEM/F12 (to remove any residual acutase), re-suspended in DMEM/F12 and counted on a hemocytometer. Four counts per treatment group per bull were evaluated. Values were expressed normalized to each bull’s control (no exogenous hormones) group.

5.4.8 Evaluating cell proliferation

Cell proliferation was evaluated using the Click-it EdU cell proliferation assay (Invitrogen) as described (Salic and Mitchison 2008). Cells were exposed to EdU (10 µM) for 18 h and then the cells were washed and fixed with 4% PFA. Three bulls were analyzed for cell proliferation. At least 1000 cells per bull per treatment group were counted. Values were expressed as a percentage of proliferating cells, normalized to each bull’s control (no exogenous hormones) group.

5.4.9 Statistical analyses

Statistical analyses are conducted using SPSS Version 22 (IBM SPSS Statistics for Macintosh, Version 22.0, IBM Corp., Armonk, NY, USA). One-way ANOVA (with post-hoc LSD test) was used to determine and locate treatment effects on cell number and percent proliferation. Data were reported as means (± SEM).
5.5 Results

5.5.1 Sertoli cell number and viability

Sertoli cell number was determined prior to cell plating. Approximately 2% of isolated cells failed to exclude trypan blue, implying that they were not viable.

Phase-contrast images of typical cells in culture are shown in Figure 5-5. These bovine Sertoli cells in culture had typical morphology by Day 1, and were spindle-shaped, elongated and proliferated by Day 7.
Figure 5-5. Phase contrast images of Sertoli cells in culture.

Phase contrast images of bovine Sertoli cells on Days 1, 4 or 7 of cell culture at two magnifications (100X and 200X)
5.5.2 Purity

Based on immunofluorescence, 96.1% ± 1.2 (mean ± SEM) of cells expressed GATA4. A representative image of GATA4 immunofluorescence is shown in Figure 5-6. Furthermore cells were negative for 3β-HSD (a known marker for Leydig cells) immunofluorescence on Day 5 (Figure 5-8). Secondary antibody controls were negative as were the BAE cells. Western blot analysis confirmed immunofluorescence results by demonstrating expression of GATA4 (Figure 5-7; 50 kDa band), but absence of 3β-HSD (results not shown) in protein extracts prepared from Sertoli cells.

Figure 5-6. Representative immunocytochemistry images of Sertoli cells stained for GATA4 and DAPI. Sertoli cells isolated from 8wk-old Holstein bull calves immunostained on Day 5 of cell culture for GATA4 and DAPI (Sertoli cell and nuclear markers, respectively).
Figure 5-7: Western blot of GATA4 (45kDA) from bovine Sertoli cells.

Figure 5-8. Representative immunocytochemistry images of Sertoli cells immunostained for 3β-HSD and DAPI.
Sertoli cells isolated from 8 wk-old Holstein bull calves immunostained on Day 5 of cell culture for 3β-HSD (Leydig cell marker; left panel), and DAPI (nuclear marker; right panel).

5.5.3 Cell numbers after IGF-I treatment

Cell numbers following hormone treatment are shown in Figure 5-9. Both IGF-I 20 & FSH and IGF-I 300 & FSH resulted in more cells than cultures treated with either FSH alone or no exogenous hormones (P < 0.05), with 1.5- and 1.4-degree fold changes, respectively.
Figure 5-9: Mean (± SEM) Sertoli cell number after 24 h of hormone treatment.
Day 7 Sertoli cell cultures were subjected to hormone treatments for 24 h. Sertoli cell numbers were determined by hemocytometer and normalized to each bulls’ control group.

Treatment groups without a common letter differed (P < 0.05).

5.5.4 Percentage of Sertoli cells expressing proliferation marker in response to IGF-I treatment

Percentages of proliferating cells following treatments are shown in Figure 5-10. The IGF-I 20, IGF-I 20 & FSH and IGF-I 300 & FSH treatment groups all had more (P < 0.05) proliferating cells than the FSH treatment group alone or the no-hormone treatment group (the latter was the basis of comparison).
Figure 5-10: Mean (± SEM) percentage of Sertoli cells expressing proliferation marker after 24 h of hormone treatment.

Day 7 Sertoli cell cultures were subjected to hormone treatments for 24 h. Cell proliferation was analyzed by EdU staining and normalized to each bulls’ control group.

\textsuperscript{a,b} Treatment groups without a common letter differed (P < 0.05).

5.6 Discussion

In many studies, Sertoli cells were isolated from rats and mice of various ages (Anway \textit{et al.} 2003; Villalpando \textit{et al.} 2008; Chang \textit{et al.} 2011). However, to our knowledge, few studies have been undertaken to isolate bovine Sertoli cells. Previous work in this area used abattoir-derived samples from bulls up to 1 y of age (Oliveira \textit{et al.} 2009); however, these bulls would be expected to be puberal and therefore, the Sertoli cell population would be very close to maturity.
In that regard, various breeds of *Bos taurus* bulls reached puberty, on average, at ~41 wk (Wolf *et al.* 1965; Almquist and Barber 1974; Amann 1983). The ability to isolate Sertoli cells from younger animals will enable researchers to investigate mechanisms modulating Sertoli cell maturation in bull calves.

Our isolation procedure, adapted from previous reports (Anway *et al.* 2003; Oliveira *et al.* 2009; Chang *et al.* 2011), used a series of enzymatic digestions (collagenase, trypsin and hyaluronidase). It was noteworthy that we were able to isolate individual cells (after passing them through cell strainers) rather than culturing “clusters of Sertoli cells” as previously described for isolating Sertoli cells from bovine testes (Oliveira *et al.* 2009). Furthermore, our cells were cultured without serum or insulin, enabling the study of bovine Sertoli cells and effects of various hormones and small molecules, without the confounding effects of other molecules being present.

Our second objective was to determine effects of IGF-I on bovine Sertoli cells. In rodents, IGF-I increases Sertoli cell proliferation and IGF-I is critical for a normal Sertoli cell population (Froment *et al.* 2007; Villalpando *et al.* 2008). However, data presented herein was apparently the first report that IGF-I also stimulated proliferation and therefore increased numbers of bovine Sertoli cells in culture.

In immature rodent Sertoli cells, FSH requires insulin/IGF signaling pathways to enhance Sertoli cell proliferation (Khan *et al.* 2002; Pitetti *et al.* 2013), consistent with our results where the combination of FSH and IGF-I yielded higher proliferation rates and more cells than either hormone alone. In rats, IGF-I had maximum stimulatory effects at concentrations between 10 and 20 ng/mL (Khan *et al.* 2002). In the present study, 20 and 300 ng/mL of IGF-I were used, based on our previous experiment (Dance *et al.* 2015) where a serum concentration of 20 ng/mL
IGF-I was physiological at 8 wk of age, and 300 ng/mL was towards maximum serum concentrations in calves during testicular development at 27 to 31 wk. It is noteworthy that these were systemic (jugular vein) concentrations, and did not take into account any locally produced IGF-I. In that regard, there is synthesis of IGF-I in somatic cells within rodent testes (Cailleau et al. 1990; Wang and Hardy 2004). Regardless, in the apparent absence of published data on concentrations of IGF-I in the bovine testes during development, these two concentrations were expected to represent a physiological range to evaluate effects of IGF-I on testicular development. Both concentrations of IGF-I with FSH increased Sertoli cell proliferation (evaluated based on expression of the proliferation marker) compared to FSH alone or no exogenous hormones (the group against which all others were normalized). Furthermore, IGF-I alone at either concentration did not increase cell number significantly, although the IGF-I 20 treatment group had significantly more cell expressing proliferation marker (EdU) than the FSH group or no hormone control. It is not clear why the IGF-I 300 ng/mL group did not also have a higher proliferation rate; perhaps the 300 ng/mL concentration was not physiological at this age and therefore, resulted in a highly variable response. However, a combination of IGF-I (either concentration) and FSH produced the most significant increase in percentage proliferation and therefore cell number. These results were consistent with rodent data suggesting that FSH amplified IGF-I effects on Sertoli cell proliferation.

Bull calves fed higher levels of both energy and protein during early-life had higher circulating IGF-I concentrations from 11 to 31 wk and ultimately had larger testes and earlier puberty (Dance et al. 2015). Therefore, in high-nutrition bulls, IGF-I may have a critical role in hastening testes maturation (and puberty). The current study certainly added validity to this argument, as IGF-I, in combination with FSH, stimulated bovine Sertoli cells to increase their
proliferation. Regardless, further research needs to be done to determine whether effects of IGF-I on proliferation of Sertoli cells in bulls operates via the same mechanisms as in rodents. In the latter, IGF-I action is mediated primarily via the type I IGF-IR, a member of the insulin receptor family of tyrosine kinase receptors (Griffeth et al. 2014). Activation of this receptor subsequently activates multiple pathways known to exert the proliferative effects of IGF-I, including PI3K/AKT and MAPK pathways (Butler et al. 1998). It is well established that FSH acts via a G protein-coupled receptor; furthermore actions of IGF-I and FSH seemed to be interconnected through common downstream signaling pathways (Griffeth et al. 2014). In an IGF-IR Sertoli cell-specific knockout study in mice, FSH was not able to exert its proliferative effects on immature Sertoli cells, suggesting that FSH required the IGF-I signaling pathway to mediate its effects (Pitetti et al. 2013). In rodent Sertoli cells, FSH amplified IGF-I mediated signaling through the PI3K/AKT pathway. Furthermore, AKT, in the PI3K pathway, has been suggested as the location where FSH and IGF-I-mediated effects interacted (Pitetti et al. 2013).

In conclusion, Sertoli cells were successfully isolated from 8 wk-old Holstein bull calves and these cells were cultured up to 8 d. A combination of IGF-I and FSH increased proliferation and therefore cell number in bovine Sertoli cells in culture, providing clear evidence that IGF-I had a similar role in bovine Sertoli cells, as reported in rodents.
Chapter Six: General discussion and future directions

The general aim of this thesis was to determine effects of early-life nutrition on reproductive performance of Holstein bulls. Currently, there is no general agreement in the Canadian AI industry regarding how to manage young bulls prior to arrival at an AI center. Regardless, the AI industry aims to maximize genetic quality and quantity of saleable semen. They also strive to reduce the interval between generations, which hastens dissemination of genetics and therefore, genetic improvement. Increased use of genomic selection for sire selection further increases the pressure to produce marketable semen from young bulls earlier. Bulls that produce more semen and are younger at puberty are therefore beneficial. Consequently, the findings of this thesis will be of interest to the AI industry.

First, I investigated effects of early-life nutrition on post-pubertal reproductive characteristics of dairy bulls. In bulls fed high-nutrition diets, endocrine profiles were enhanced, testes size increased and age at puberty decreased.

The diet manipulations in this study focused on varying levels of energy and protein. There has been increasing evidence however, that the type of fatty acids contained in the diets plays an important role in male and female reproduction. Polyunsaturated fatty acids are necessary for numerous processes in the body, but specifically for reproduction, they provide the precursors for prostaglandin synthesis, and are involved in steroid metabolism by modulating the enzymes involved (Wathes et al. 2007). In females, supplementing the diets with fish oils containing high polyunsaturated fatty acids improved conception rates (Burke et al. 1997). Additionally, supplementation with alpha-linolenic acid (a precursor to important fatty acids) lowered pregnancy losses in Holstein cows (Ambrose et al. 2006). In males, unsaturated fatty acids are critical for a proper functioning sperm plasma membrane, as they give the membrane
the fluidity it needs for the events surrounding fertilization (Wathes et al. 2007). However, it appears that little work has been done to determine the effects of dietary fatty acids during reproductive development in bull calves. In the current study we did not specifically measure the fatty acid composition of the diets in this experiment, however our diets did not contain grains that are considered oilseed crops such as flax and sunflower seeds, which are known to provide high levels of alpha-linoleic acid and linoleic acid respectively to the animals (Colazo et al. 2004). Further work should be done to determine how important the level of energy and protein is compared to the composition of the diets (in terms of fatty acids) to the reproductive development of Holstein bull calves, and if similar results could be achieved by altering fatty acid content as we have shown in this study.

A key finding from the first study was that IGF-I concentrations were significantly increased in high- versus low-nutrition bulls. Several interesting questions arose from these findings. We focused on exploring effects of early-life nutrition modulation on reproductive characteristics of these bulls. Although several differences were identified, further work is required to determine mechanisms by which different diets are affecting hormone concentrations and reproductive development and what truly is the first “signal” to the body to increase nutrients allotted for reproduction. In this study, we coupled early-life milk feeding modulation with post weaning diet modulations. Further work should be completed to uncouple these two manipulations and determine if one of them has a more pronounced effect on reproductive development than the other. Differences in early-life milk diets in Holstein calves have been shown to alter growth parameters (Hill et al. 2008; Diaz et al. 2001) whereas in the previous beef bull studies, only post-weaning dietary manipulation were evaluated (Brito et al. 2007a; Brito et al. 2007c). In the current study, we saw slightly more pronounced differences in reproductive
characteristics then were observed in the beef bull studies. This could be due to the addition of the milk diet modulation or due to breed differences (beef vs. dairy). Over the years, beef and dairy animals have been selected for very different characteristics and have been managed differently. As a result, the way they respond to early-life dietary changes could be very different. This was one of the main drivers for our study, to begin by repeating Brito et al.’s work with beef bulls, to determine if similar responses to nutritional modulation were observed with dairy bulls.

Based on increased IGF-I concentrations, the role of IGF-I was pursued in subsequent experiments. Regardless, other molecules could also be involved in this process and further study is needed to determine exact mechanisms.

It is known that IGF-I is under control of GH, mimics GH concentrations systemically, and that much of GH’s systemic effects are dependent on expression of IGF-I (Le Roith et al. 2001). Growth hormone (GH) is released in pulses from the pituitary; these pulses are critical for postnatal growth and in particular for the “accelerated growth seen during the peri-pubertal period” (Le Roith et al. 2001). In addition to IGF-I, GH regulates gene expression of many other growth factors, including fibroblast growth factor, hepatocyte growth factor, EGF, and estrogen receptors (Ekberg et al. 1989; Bezecny et al. 1992). Perhaps one or more of these growth factors are contributing to the observed effects. Additionally, there could be other factor(s) outside the GH and IGF-I system that also contributed to enhanced reproductive development.

In the present study, enhanced nutrition from 2 to 31 wk had profound positive effects on reproductive development and sperm production. These findings are of interest to the dairy industry, as they provided insight into how better nutritional management of young bulls during early-life could increase reproductive potential. Based on our data, AI centres should consider
creating an optimized nutritional program for their young bull calves starting at a much younger age than previously anticipated in order to best maximize bull reproductive potential. This must be done early in life, as in beef bulls, better nutrition after 31 wk did not overcome the negative effects of poor nutrition prior to 31 wk (Brito et al. 2007a). Regardless, it would be helpful to better define the exact age and duration required for the nutritional modulation, to both maximize reproductive potential and minimize input costs.

One of the main questions that arises from this portion of the study was: Does early-life nutritional modulation have a negative effect on semen quality in adult bulls? It is important to the AI industry that this question is addressed, as the benefits of the early-life nutrition would be negated if semen quality was impaired. Another important question for the AI industry was: What is the cost-benefit ratio of early-life nutritional modulation? Therefore, we investigated semen quality by evaluating sperm characteristics (viability, protein profiles and fertility in vitro). There were no significant differences in semen quality of bulls across treatment groups. Furthermore, the cost differential of the high- versus low-nutrition diet was negligible in comparison to the extra income that could be gained from the additional sperm produced by high-nutrition fed bulls. These findings reinforced the recommendation to the AI industry to begin implementing higher-nutrition diets in their young bull sires during early-life. Due to the relatively low costs of feeding the younger bulls the high-nutrition diet, many bulls could be put in such a program, even though not all are destined to become top AI sires. Consequently, there is no need to select sires prior to implementing early-life nutritional modifications.

The results of these studies were extremely relevant for the AI industry; however, they also brought up some more basic science questions. The next question that emerged was what was happening at the testicular level during the differential feeding period to cause differences in
adult bulls. To address that, we castrated bull calves at four ages during the differential feeding period to determine what was happening at the testicular level. From this study, we concluded that 24 wk appeared to be a critical time of development in Holstein bull calves, as nutritional modulation caused significant differences among treatment groups at this age. However, it is important to note that we investigated these calves at fairly wide intervals (8, 16, 24, and 32 wk) in this study. Further work should be done to more critically investigate the time from 16-24 wk to determine when the differences in testes development between the treatment groups is really manifesting. For this reason, we can’t not definitively say that 24 wk is the time when the testicular differences between high- and low-nutrition calves are beginning. 24 wk could be used as a target time to evaluate bulls for early versus late maturation. Determining what testes size qualifies as early vs late maturing at this age would require further investigation.

We concluded that higher early-life nutrition did not alter efficiency of spermatogenesis or efficiency of Sertoli cells to support developing germ cells; however, it increased total number of Sertoli cells, resulting in longer tubules and larger testes. Additionally, high-nutrition calves also had more mature Sertoli cells earlier than low-nutrition calves, consistent with their earlier puberty. Mature Sertoli cell markers were expressed at higher levels in high-nutrition calves at 24 wk and there was also more proliferative cells in their testes. This suggests that better early-life nutrition triggered more cellular proliferation in the testes, resulting in more Sertoli cells. In this study, we use whole testis extracts to evaluate Sertoli cell and proliferation markers. This provides some limitations to the results, as these markers could be expressed by any of the cells present in the testes. Future work should consider uncoupling each of the cell populations and evaluating expression of proliferation and maturation markers independently of the other cell populations to get a more accurate representation of what is occurring in the testes during the
nutritional modulation. We have shown an important first step however, that early-life nutritional modulation is affecting the proliferation and maturation of the cells within the testis.

In this study as well as the first study, IGF-I was differentially expressed between high- and low-nutrition bulls. This led to the question: what was the role of IGF-I during maturation of bovine testes? Therefore, we explored direct effects of IGF-I on bovine Sertoli cells in culture. In that study, IGF-I, in combination with FSH, stimulated bovine Sertoli cells to increase their proliferation. In addition, Sertoli cells were successfully isolated from 8 wk-old Holstein bull calves. A combination of IGF-I and FSH successfully increased proliferation and therefore cell number in bovine Sertoli cells in culture, providing clear evidence that IGF-I had a critical role in reproductive development of bull calves. Further studies are required to understand the signaling pathways associated with this increased proliferation, and to determine if FSH and IGF-I are acting via the same mechanisms they are known to in rodents.

In this thesis, I focused on increased systemic production of IGF-I and its effects on male reproductive characteristics. However, it is noteworthy that local testes production of many hormones including IGF-I and GnRH has been documented. The effects that early-life nutrition could be having on this local environment have not been investigated and are an area where further research is required.

More research is also needed to be done to determine how effects of IGF-I on Sertoli cells proliferation can be translated into a management tool for the AI industry. Perhaps it is possible to feed diets that selectively up-regulate IGF-I. Alternatively, perhaps giving young bull calves IGF-I-releasing implants would have the same effects on reproductive development induced by an enhanced diet?
Nutrition has been shown to alter the epigenetic regulation of some genes (Anderson et al. 2012). Epigenetic changes to the DNA are heritable, and are a molecular modification that does not alter the DNA sequence but can alter gene expression (Li 2002). These modifications have been shown to be influenced by a wide variety of external factors (Jirtle and Skinner 2007). In particular, early-life and maternal-nutrition has been shown to change the epigenetic characteristics of the offspring and as a result, can have profound influence on the phenotype of adult animal and their health status (Vickers 2014, Waterland and Jirtle 2004). Determining if epigenetics is somehow playing a role in the observed early-life nutritional modulation effects on Holstein bull reproductive characteristics would be extremely interesting and add substantially to the understanding of the nutritional mechanisms affecting reproductive potential.

6.1 Conclusions

Overall, bulls fed a high-nutrition diet from 2 to 31 wk were younger at puberty, had larger testes and had higher concentrations of circulating IGF-I than low-nutrition bulls. Furthermore, high-nutrition bulls had more proliferating and differentiating Sertoli cells earlier in life than low-nutrition bulls and IGF-I promoted proliferation of bovine Sertoli cells in culture. Overall, we determined that IGF-I had important roles in bovine reproduction and we identified key periods during bull development where it appeared that nutritional modulation was affecting testicular development.

Determining that better early nutrition results in larger testis with greater sperm production are expected to increase profits for the AI industry. Also, if this management technique hastens puberty, bulls deemed genetically superior will begin producing semen earlier, facilitating faster production of genetically superior offspring. Therefore, this research will have
wide-spread implications for the Canadian dairy industry. Elucidating reproductive mechanisms
behind these improvements in pubertal age and testicular size will also benefit the scientific
community. Understanding why there is a critical period for nutrition in bulls and the effects on
the animal, with respect to hormone concentrations and characteristics of testes or semen, will
generate new knowledge regarding underlying mechanisms, and should stimulate further
research in this field. Moreover, results of this study validate nutritional modulation as a model
for studying male reproduction.
References

Abdel-Raouf, M. (1960) The postnatal development of the reproductive organs in bulls with special reference to puberty (including growth of the hypophysis and the adrenals). *Acta Endocrinol Suppl (Copenh) 34(Suppl 49), 1-109*


APPENDIX A: PRELIMINARY CELL CULTURE EXPERIMENTS

A.1. Materials and methods

A.1.1. Bulls

Holstein bull calves (n = 4) were castrated at 8, 16, 24 and 32 wk as described in Chapter 5. This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee.

A.1.2. Cell isolation

Sertoli cells were isolated as described in Chapter 5.

A.1.3. Cell culture and hormone treatments

The 8 wk-old cells isolated here were first cultured with ITS (Sigma) in the culture media. On Day 7 of culture, cells were treated with recombinant human IGF-I (291-G1, R&D Systems; 20 ng/mL), FSH (bovine FSH, Sioux Pharmaceuticals; 0.4 ng/mL), IGF-I and FSH together (20 ng/mL and 0.4 ng/mL, respectively), or no exogenous hormones (control). After culturing these cells and obtaining the results, it was decided that the insulin in the ITS could be confounding our IGF-I proliferation results; therefore, we decided to proceed without insulin in the culture media for the other age groups.

Cells isolated from 16 wk-old calves were treated the same as the 8 wk-old cells, except for the removal of the insulin from the culture system. For the cells isolated from the 24 wk and 32 wk-old calves, higher concentrations of IGF-I were added as treatment groups as by these ages physiologically, the 20 ng/mL was too low. Therefore, on Day 7 of culture, cells were
treated with recombinant human IGF-I (291-G1, R&D Systems; 20, 150 or 300 ng/mL), FSH (bovine FSH, Sioux Pharmaceuticals; 0.4 ng/mL), IGF-I and FSH together (20 ng/mL and 0.4 ng/mL, respectively), or no exogenous hormones (control). For all age groups, after 24 h of exposure to hormones, cells were evaluated for cell number or expression of EdU to signify proliferation, as described in Chapter 5.

**A.1.4. Statistical analyses**

Statistical analyses are conducted using SPSS Version 22 (IBM SPSS Statistics for Macintosh, Version 22.0., IBM Corp., Armonk, NY, USA). One-way ANOVA (with post-hoc LSD test) was used to determine and locate treatment effects on percent proliferation with in an age group. Data were reported as means (± SEM).

**A.2. Results**

Percentage of proliferating cells following treatment are shown in Figure B-1. At 16 wk, the IGF-I 20 and FSH treatment group had more (P < 0.05) proliferating cells than the IGF-I 20 or FSH groups alone. At 32 wk, the IGF-I 300 group had more (P < 0.05) proliferating cells than all the other treatment groups.
Figure B-1. Mean (± SEM) percentage of Sertoli cells expressing proliferation marker after 24 h of hormone treatment.

Day 7 Sertoli cell cultures were subjected to hormone treatments for 24 h. Cell proliferation was analyzed by EdU staining and normalized to each bulls’ control group. Treatment groups with an asterisk differ from the others (P < 0.05).

A.3. Discussion

Data from this experiment demonstrated that IGF-I is stimulating Sertoli cell proliferation. This is consistent with our hypothesis and all other data to date. However, for this experiment and given the treatment groups that were used, the effect of IGF-I on Sertoli cell proliferation was not entirely clear at all age groups. We were missing the higher IGF-I concentration treatment group in the 8 and 16 wk groups for comparison and we were missing the combinations of FSH with IGF-I 300 across all age groups.

For these reasons, we decided to focus on the 20 and 300 ng/mL IGF-I concentrations and apply both of these IGF-I concentrations in combination with FSH. We were unable to
obtain testes from all of the age periods therefore we completed these treatment groups on Sertoli cells isolated from 8 wk-old calves (Chapter 5).

Data presented in this appendix were consistent with the complete data in Chapter 5. IGF-I increased cell proliferation and FSH and IGF-I together had an even greater effect on cell proliferation. We hypothesize that in 32 wk-old animals, we would have observed significantly higher proliferation with the IGF-I 300 & FSH treatment compared to the FSH treatment alone. This will require further investigation to confirm, however this trial allowed us to refine our techniques and treatment groups for experiments in Chapter 5.
APPENDIX B: COPYRIGHT PERMISSIONS

B.1. Co-Author release letter

Dear Dr. Jacob Thundathil/ Dr. John Kastelic

I would like to inform you that work we have collaborated on, in which you are a co-author, has been included in my PhD thesis and will be submitted to Library and Archives Canada via The University of Calgary. As a part of this required submission, I have signed a Theses Non-Exclusive Licence that authorizes Library and Archives Canada to reproduce, communicate to the public on the Internet, loan, distribute or sell copies of my thesis among other things.

As you are co-author on the manuscripts listed below that have been submitted for publication or published. I am requested to obtain your permission to submit my thesis for publication via Library and Archives Canada, and the aforementioned licence agreement. If you consent to this, please return a signed copy of the attached letter to me at your earliest convenience.

I appreciate all of your assistance and guidance in the process of obtaining my PhD.

Regards,

Alysha Dance
I, ____________________________, consent to the inclusion of the following manuscripts that I have co-authored in the PhD thesis of Alysha Dance for publication with Library and Archives Canada, including the agreements included in the Thesis Non-Exclusive Licence that authorizes Library and Archives Canada to reproduce, communicate to the public on the Internet, load, distribute or sell copies of the thesis, among other things.


Submitted to Reprod Fertil Dev

Regards,

Signature                           Print Name                           Date
B.2. Journal of Dairy Science release

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jun 07, 2016

This Agreement between Alysha Dance ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number 382550804656
License date Mar 08, 2016
Licensed Content Publisher Elsevier
Licensed Content Publication Journal of Dairy Science
Licensed Content Title Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls
Licensed Content Author Alysha Dance, Jacob Thundathil, Randy Wilde, Patrick Blondin, John Kastelic
Licensed Content Date February 2015
Licensed Content Volume Number 98
Licensed Content Issue Number 2
Start Page 987
End Page 998
Type of Use reuse in a thesis/dissertation
Portion full article
Format both print and electronic
Are you the author of this Elsevier article? Yes
Will you be translating? No
<table>
<thead>
<tr>
<th>Order reference number</th>
<th>Nutritional modulation of reproductive potential in dairy bulls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title of your thesis/dissertation</td>
<td>Apr 2016</td>
</tr>
<tr>
<td>Expected completion date</td>
<td></td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td></td>
</tr>
<tr>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td>Requestor Location</td>
<td>Alysha Dance 1404 3500 Varsity Dr. NW</td>
</tr>
</tbody>
</table>

Calgary, AB T2L 1Y3
Canada
Attn: Alysha Dance

| Billing Type | Invoice |
| Billing Address | Alysha Dance 1404 3500 Varsity Dr. NW |

Calgary, AB T2L 1Y3
Canada
Attn: Alysha Dance

Total | 0.00 CAD |

Terms and Conditions

INTRODUCTION
1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions
and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.
LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world *English* rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx) or the Elsevier homepage for books at[http://www.elsevier.com](http://www.elsevier.com); Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at [http://www.elsevier.com](http://www.elsevier.com). All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors**: the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts**: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement

- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for
classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation:** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at [http://creativecommons.org/licenses/by/4.0](http://creativecommons.org/licenses/by/4.0).

**CC BY NC SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not
done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

**CC BY NC ND**: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:
B.3. Theriogenology release

This is a License Agreement between Alysha Dance ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier

Elsevier Limited
The Boulevard, Langford Lane
Kidlington, Oxford, OX5 1GB, UK

Registered Company Number

1982084

Customer name

Alysha Dance

Customer address

1404 3500 Varsity Dr. NW
Calgary, AB T2L 1Y3

License number

3857951047182

License date

Apr 20, 2016

Licensed content publisher

Elsevier

Licensed content publication

Theriogenology

Licensed content title

Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing post-pubertal semen quality

Licensed content author

Alysha Dance, Jacob Thundathil, Patrick Blondin, John Kastelic

Licensed content date

Available online 4 March 2016

Licensed content volume number

n/a

Licensed content issue number

n/a

Number of pages

1

Start Page

None

End Page

None

Type of Use

reuse in a thesis/dissertation
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought
from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world [English] rights only unless your license was granted for translation rights. If you licensed translation rights you may only
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors**: the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version.
Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- **Immediately**
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement

- **After the embargo period**
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation:** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
**CC BY NC ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at [http://creativecommons.org/licenses/by-nc-nd/4.0](http://creativecommons.org/licenses/by-nc-nd/4.0). Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- ☐ Associating advertising with the full text of the Article
- ☐ Charging fees for document delivery or access
- ☐ Article aggregation
- ☐ Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions:**