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In utero and in vitro sex ratio of bovine embryos and calves originating from the left and right ovaries

Darin Alan Hylan

Louisiana State University and Agricultural and Mechanical College

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IN UTERO AND IN VITRO SEX RATIO OF BOVINE EMBRYOS
AND CALVES ORIGINATING FROM THE LEFT AND RIGHT OVARIES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Animal and Dairy Sciences

by
Darin Alan Hylan
B.S., Louisiana Tech University, 1995
M.S., Louisiana State University, 2000
May 2007
THIS DISSERTATION IS DEDICATED TO THE MEMORY OF MY GODPARENTS, LELAND TEAGUE AND HAZEL VIOLA TEAGUE, WHO ALWAYS REMINDED ME OF THE IMPORTANCE OF AN EDUCATION.
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To the author’s family, who has also experienced the highs and lows associated with graduate study, the author gives sincere appreciation. Words on a piece of paper simply cannot express the value and importance of their unwavering support through the years, both before and during the author’s graduate studies.

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# TABLE OF CONTENTS

**DEDICATION** ..................................................................................................................... ii  

**ACKNOWLEDGMENTS** ....................................................................................................... iii  

**LIST OF TABLES** ............................................................................................................. ix  

**LIST OF FIGURES** .......................................................................................................... x  

**LIST OF ABBREVIATIONS** .............................................................................................. xiv  

**STANDARDIZED TERMINOLOGY** ..................................................................................... xvi  

**ABSTRACT** ........................................................................................................................ xvii  

**CHAPTER**  

I. **INTRODUCTION** ........................................................................................................... 1  

II. **REVIEW OF LITERATURE** .......................................................................................... 4  
   Theories of Maternal Control of Offspring Sex ............................................................... 4  
   Theory of Declining Maternal Conditions ................................................................. 4  
   Societal Support Theories ......................................................................................... 6  
   Sibling Competition for Mates or Resources ......................................................... 6  
   Parental Competition ............................................................................................. 7  
   Sibling Cooperation ............................................................................................... 7  
   Parental Cooperation .......................................................................................... 8  
   Juvenile Mortality During Parental Investment .................................................... 8  
   Fluctuations in the Adult Sex Ratio ................................................................. 9  
   Inbreeding ............................................................................................................... 9  
   Influences on Sex Ratio Variation ....................................................................... 10  
   Litter Size ............................................................................................................. 10  
   Maternal Age ...................................................................................................... 11  
   Parity of the Mother .......................................................................................... 12  
   Maternal Fecundity ............................................................................................ 13  
   Nutrition ............................................................................................................ 13  
   Stress ................................................................................................................ 15  
   Habitat Quality .................................................................................................. 16  
   Population Demography .................................................................................. 16  
   Social Dominance Order ............................................................................... 18  
   Climatic Variation .......................................................................................... 20  
   Season of Birth .................................................................................................. 21  
   Timing of Insemination ..................................................................................... 21
III. DISTRIBUTION OF SEXES WITHIN THE LEFT AND RIGHT UTERINE HORNS OF BEEF CATTLE

Introduction ........................................................................................................... 31
Materials and Methods ......................................................................................... 32
Experiment 3.1 ...................................................................................................... 32
Collection of Reproductive Tracts ......................................................................... 32
Horn of Gestation Verification .............................................................................. 33
Sex Determination ................................................................................................. 33
Experiment 3.2 ...................................................................................................... 33
Experimental Animals ......................................................................................... 33
Synchronization and Insemination Protocol .......................................................... 34
Pregnancy and Sex Determination ......................................................................... 34
Experiment 3.3 ...................................................................................................... 35
Experimental Animals ......................................................................................... 35
Pregnancy and Sex Determination ......................................................................... 35
Statistical Analysis ................................................................................................. 35
Results ................................................................................................................... 38
Experiment 3.1 .................................................................................................. 38
Experiment 3.2 .................................................................................................. 38
Experiment 3.3 .................................................................................................. 47
Combined Experiments ......................................................................................... 53
Discussion .............................................................................................................. 53

IV. SEX RATIO OF EMBRYO TRANSFER CALVES PRODUCED FROM EMBRYOS RECOVERED FROM SUPEROVULATED DONORS AND TRANSFERRED TO THE LEFT AND RIGHT UTERINE HORNS OF RECIPIENTS

Introduction .......................................................................................................... 61
Materials and Methods .......................................................................................... 62
Experimental Animals .......................................................................................... 62
Superovulation Protocol ......................................................................................... 62
Embryo Collection Procedure .............................................................................. 65
Embryo Transfer ................................................................................................. 66
Statistical Analysis ................................................................................................. 66
Results ................................................................................................................... 67
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. SEX RATIO OF EMBRYOS RECOVERED FROM THE LEFT AND RIGHT UTERINE HORNS OF SUPEROVULATED BEEF COWS</td>
<td>73</td>
</tr>
<tr>
<td>Introduction</td>
<td>73</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>74</td>
</tr>
<tr>
<td>Experimental Animals</td>
<td>74</td>
</tr>
<tr>
<td>Superovulation Protocol</td>
<td>74</td>
</tr>
<tr>
<td>Embryo Collection Procedure</td>
<td>76</td>
</tr>
<tr>
<td>Embryo Sex Determination</td>
<td>79</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>80</td>
</tr>
<tr>
<td>Results</td>
<td>82</td>
</tr>
<tr>
<td>Discussion</td>
<td>90</td>
</tr>
<tr>
<td>VI. SEX RATIO OF EMBRYOS RECOVERED FROM UNILATERALLY-OVARIECTOMIZED, SUPEROVULATED BEEF HEIFERS</td>
<td>94</td>
</tr>
<tr>
<td>Introduction</td>
<td>94</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>95</td>
</tr>
<tr>
<td>Experimental Animals</td>
<td>95</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>96</td>
</tr>
<tr>
<td>Superovulation Protocol</td>
<td>96</td>
</tr>
<tr>
<td>Embryo Collection Procedure</td>
<td>98</td>
</tr>
<tr>
<td>Embryo Sex Determination</td>
<td>100</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>101</td>
</tr>
<tr>
<td>Results</td>
<td>102</td>
</tr>
<tr>
<td>Discussion</td>
<td>117</td>
</tr>
<tr>
<td>VII. SEX RATIO OF IN VITRO BOVINE EMBRYOS RESULTING FROM ABATTOIR-DERIVED OOCYTES ORIGINATING FROM</td>
<td>119</td>
</tr>
<tr>
<td>THE LEFT AND RIGHT OVARIRES</td>
<td>119</td>
</tr>
<tr>
<td>Introduction</td>
<td>119</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>120</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>120</td>
</tr>
<tr>
<td>Experiment 7.1</td>
<td>120</td>
</tr>
<tr>
<td>Experiment 7.2</td>
<td>120</td>
</tr>
<tr>
<td>Ovary Collection</td>
<td>121</td>
</tr>
<tr>
<td>Oocyte Aspiration</td>
<td>121</td>
</tr>
<tr>
<td>Maturation of Oocytes</td>
<td>122</td>
</tr>
<tr>
<td>IVF Media Preparation</td>
<td>122</td>
</tr>
<tr>
<td>Semen Preparation</td>
<td>122</td>
</tr>
<tr>
<td>In Vitro Fertilization</td>
<td>123</td>
</tr>
</tbody>
</table>
In Vitro Culture .............................................................................................. 124
Embryo Development .................................................................................. 125
Embryo Sex Determination ....................................................................... 126
Statistical Analysis ...................................................................................... 127
Results ............................................................................................................ 127
Experiment 7.1 ............................................................................................. 127
Experiment 7.2 ............................................................................................. 135
Discussion ..................................................................................................... 153

VIII. SUMMARY AND CONCLUSIONS ................................................................. 159

LITERATURE CITED ....................................................................................... 162

APPENDIX: SUPPLEMENTAL MEDIA AND STOCK SOLUTIONS ......................... 194

VITA .................................................................................................................. 205
LIST OF TABLES

3.1 Distribution of male and female fetuses between the left and right uterine horns in gravid, slaughterhouse-derived reproductive tracts...........................................39

3.2 Distribution of male and female calves gestated in the left and right uterine horns of artificially inseminated heifers ...........................................................................42

3.3 Distribution of male and female calves gestated in the left and right uterine horns of naturally mated beef cows.................................................................................48

3.4 Combined distribution of male and female pregnancies gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3.........................................................55

4.1 Distribution of male and female calves resulting from the transfer of embryos to the left and right uterine horns of recipients ..............................................................68

5.1 The modified Ryan Embryo Development (mRED) rating system ........................................78

5.2 Distribution of male and female embryos collected from the right and left uterine horns of superovulated beef cows.................................................................83

5.3 Mean (±SEM) mRED Score for male and female embryos recovered from the left and right uterine horns of superovulated beef cows......................................................88

6.1 Distribution of male and female embryos collected from the right and left uterine horns of unilaterally ovariectomized, superovulated beef heifers.................................103

6.2 Mean (±SEM) mRED Score for male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers .........................110

7.1 Distribution of male and female embryos originating from oocytes derived from the left and right ovaries..................................................................................................128

7.2 Mean (±SEM) mRED Score for male and female in vitro-derived embryos originating from the left and right ovaries ........................................................................133

7.3 Distribution of male and female embryos originating from oocytes derived from the left and right ovaries after different time periods of in vitro maturation..........................138

7.4 Mean (±SEM) mRED Score of male and female embryos originating from oocytes derived from the left and right ovaries after different periods of in vitro maturation .............................................................146
LIST OF FIGURES

3.1 Timeline of OvSynch protocol for timed insemination of beef heifers (Pursley et al., 1995) .....................................................................................................................36

3.2 Location of research cattle herds in Louisiana used for the collection of sex ratio data in Experiment 3.3 .................................................................37

3.3 Percentage of pregnancies established in the left and right uterine horns of slaughterhouse-derived reproductive tracts .................................................................40

3.4 Sex ratio (% male) of fetuses in the left and right uterine horns of slaughterhouse-derived reproductive tracts .................................................................41

3.5 Percentage of pregnancies gestated in the left and right uterine horns of artificially inseminated heifers ..................................................................................43

3.6 Sex ratio (% male) of calves in the left and right uterine horns of artificially inseminated heifers ..........................................................................................44

3.7 Percentage of male and female calves gestated in the left and right uterine horns of artificially inseminated heifers ................................................................45

3.8 Sex ratio (% male) of calves gestated in the left and right uterine horns of artificially inseminated heifers by year ........................................................................46

3.9 Percentage of pregnancies gestated in the left and right uterine horns of naturally mated, crossbred beef cows .................................................................49

3.10 Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows .................................................................50

3.11 Percentage of male and female calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows .................................................................51

3.12 Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows by year ........................................................................52

3.13 Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows by location .................................................................54

3.14 Percentage of pregnancies gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3 .........................................................................................56
3.15 Sex ratio (% male) of fetuses and calves gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3.....................................................................................................................57

4.1 Location of farms used for the collection of embryo transfer and calving data in the state of Maranhão in northeast Brazil..............................................................................................63

4.2 Timeline of the superovulation protocol for Nelore donor cattle .........................................................64

4.3 Percentage of calves resulting from embryos derived from superovulated donors and transferred to the left and right uterine horns of recipients..........................................................69

4.4 Sex ratio (% male) of calves resulting from the transfer of embryos to the left and right uterine horns of recipients............................................................................................................70

4.5 Sex ratio (% male) of calves resulting from the transfer of embryos to the left and right uterine horns of recipients by location................................................................................................71

5.1 Timeline of the superovulation protocol for crossbred beef donor cattle..............................................75

5.2 Agarose gel under UV illumination after electrophoresis for sex determination of bovine embryos .................................................................................................................................81

5.3 Percentage of embryos recovered from the left and right uterine horns of superovulated beef cows.................................................................................................................................84

5.4 Sex ratio (% male) of embryos recovered from the left and right uterine horns of superovulated cows............................................................................................................................85

5.5 Distribution of male and female embryos recovered from the left and right uterine horns of superovulated cows................................................................................................................86

5.6 Sex ratio (% male) of embryos recovered from the left and right uterine horns of superovulated beef cows by replicate ........................................................................................................87

5.7 Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of superovulated beef cows........................................................................89

5.8 Mean (±SEM) mRED Score of male and female embryos recovered from superovulated beef cows by replicate .............................................................................................................91

6.1 Timeline of the superovulation protocol for unilaterally ovariectomized beef heifers.................................97

6.2 Percentage of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers ........................................................................................................104
6.3 Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally-ovariectomized beef heifers ................................................................. 105

6.4 Percentage of male and female embryos collected from left- and right-ovary intact unilaterally ovariectomized heifers ................................................................. 106

6.5 Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by replicate ........................................ 107

6.6 Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by group ........................................ 108

6.7 Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers ......................... 111

6.8 Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by sex ............ 112

6.9 Mean (±SEM) mRED Score of embryos recovered from the left and right uterine horns of unilaterally ovariectomized heifers by replicate .................................. 113

6.10 Mean (±SEM) mRED Score of male and female embryos recovered from unilaterally ovariectomized beef heifers by replicate .................................................. 114

6.11 Mean (±SEM) mRED Score of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by group .................................. 115

6.12 Mean (±SEM) mRED Score of male and female embryos recovered from unilaterally ovariectomized beef heifers by group .................................................. 116

7.1 Sex ratio (% male) of embryos derived from oocytes originating from the left and right ovaries ........................................................................................................ 129

7.2 Percentage of male and female embryos derived from oocytes originating from the left and right ovaries ................................................................................ 131

7.3 Sex ratio (% male) by replicate of embryos derived from oocytes originating from the left and right ovaries ................................................................................ 132

7.4 Mean (±SEM) mRED Score of male and female embryos derived from oocytes originating from the left and right ovaries ......................................................... 134

7.5 Mean (±SEM) mRED Score of male and female in vitro produced embryos by replicate .............................................................................................................. 136
7.6 Mean (±SEM) mRED Score of embryos derived from oocytes originating from the left and right ovaries by replicate ................................................................. 137

7.7 Overall sex ratio (% male) of embryos originating from oocytes derived from the left and right ovaries after in vitro maturation ......................................................... 140

7.8 Distribution of male and female embryos derived from oocytes originating from the left and right ovaries after in vitro maturation .................................................. 141

7.9 Sex ratio (% male) by treatment of embryos derived from oocytes originating from the left and right ovaries after different time periods of in vitro maturation ................................................................. 142

7.10 Sex ratio (% male) by replicate of embryos derived from oocytes originating from the left and right ovaries after in vitro maturation ................................................ 143

7.11 Sex ratio (% male) by replicate of embryos after different treatment periods of in vitro maturation ............................................................................................................. 145

7.12 Mean (±SEM) mRED Score of male and female embryos derived from oocytes originating from the left and right ovaries after all time periods of in vitro maturation ...................................................................................... 147

7.13 Mean (±SEM) mRED Score of embryos originating from oocytes derived from the left and right ovaries after different time periods of in vitro maturation ................................................................. 149

7.14 Mean (±SEM) mRED Score of male and female embryos after different time periods of in vitro maturation ............................................................................................. 151

7.15 Overall mean (±SEM) mRED Score by replicate of embryos originating from the left and right ovaries after all time periods of in vitro maturation .............................. 152

7.16 Mean (±SEM) mRED Score by replicate of male and female embryos after all time periods of in vitro maturation ........................................................................................................ 154

7.17 Timeline for sex ratios (% male) of embryos derived from oocytes originating from the left and right ovaries after in vitro maturation for different periods ............................. 155

7.18 Timeline of mRED Scores for male and female embryos derived from oocytes originating from the left and right ovaries after in vitro maturation for different periods ................................................................. 156
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>Artificial Insemination</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technologies</td>
</tr>
<tr>
<td>BO</td>
<td>Brackett-Oliphant</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CIDR</td>
<td>Controlled Internal Drug Release</td>
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<tr>
<td>CA</td>
<td>Corpus Albican</td>
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<tr>
<td>CH</td>
<td>Corpus Hemorrhagicum</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus Luteum</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus-Oocyte Complex</td>
</tr>
<tr>
<td>DFR</td>
<td>Dominant Follicle Removal</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol-17β</td>
</tr>
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<td>Estradiol Benzoate</td>
</tr>
<tr>
<td>ET</td>
<td>Embryo Transfer</td>
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<tr>
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<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
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<td>ICM</td>
<td>Inner Cell Mass</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like Growth Factor-I</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IVC</td>
<td>In Vitro Culture</td>
</tr>
<tr>
<td>IVF</td>
<td>In Vitro Fertilization</td>
</tr>
<tr>
<td>IVM</td>
<td>In Vitro Maturation</td>
</tr>
<tr>
<td>IVP</td>
<td>In Vitro Production</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LN₂</td>
<td>Liquid Nitrogen</td>
</tr>
<tr>
<td>M-I</td>
<td>Metaphase-I</td>
</tr>
<tr>
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<td>Metaphase-II</td>
</tr>
<tr>
<td>P₄</td>
<td>Progesterone</td>
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<tr>
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<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGF$<em>2$$</em>\alpha$</td>
<td>Prostaglandin F$<em>2$$</em>\alpha$</td>
</tr>
<tr>
<td>QS</td>
<td>Quantum Satis/Quantum Sufficiat</td>
</tr>
<tr>
<td>RED</td>
<td>Ryan Embryo Development</td>
</tr>
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<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TCM-199</td>
<td>Tissue Culture Medium-199</td>
</tr>
<tr>
<td>TRT</td>
<td>Treatment</td>
</tr>
</tbody>
</table>
STANDARDIZED TERMINOLOGY

Sex ratio is generally defined as the proportion of males to females in a given population at a specific stage in life between conception and death and is usually expressed as the number of males per 100 females. The formal way of stating sex ratio is by separating the quantities of the two sexes with a colon (e.g., 105:100 or 1.05:1) although sometimes sex ratio is expressed as a single value (e.g., 105 or 1.05). By contrast, in the biological sciences, sex ratio is often defined as the proportion of males in a given population and is expressed as a percentage (e.g., 51.2%). In an effort to eliminate confusion, all numerical values for sex ratio in this dissertation are expressed as the percentage of males. Cited values from previously published studies originally expressed as a ratio have been converted to percent males.

In this dissertation, parity is used to express two separate connotations. First, parity is used to describe the number of offspring to which a female has given birth, and can be expressed as a noun (e.g., females with increasing parity) or as an adjective (e.g., multiparous females). In addition, parity is used to express equality in amount. Therefore, when a given sex ratio is compared with parity, the comparison is with an expected ratio of 50% or an equal number of males and females.
ABSTRACT

An asymmetric distribution of the sexes within the left and right uterine horns has been described in multiple polytocous, laboratory species. A series of experiments were conducted to evaluate the sex ratio (% male) of calves gestated in the left and right uterine horns, as well as the sex ratio of embryos originating from the left and right ovaries of cattle. In Experiment 1, the sex ratio of calves and fetuses gestated in the left and right uterine horns was investigated. The sex ratio of calves and fetuses gestated in the right uterine horn was significantly higher compared with the sex ratio of calves and fetuses gestated in the left uterine horn. In addition, the sex ratio of the left and right uterine horns differed significantly from parity. In Experiment 2, embryo transfer data were analyzed in an effort to determine if sex-specific selection pressures were applied to embryos in the uterine horn of transfer. The sex ratio of ET calves born following transfer to the left and right uterine horns was not significantly different. Similarly, the overall sex ratio of calves born in this experiment did not differ significantly from parity. The sex ratio of embryos recovered from the left and right uterine horns of superovulated beef cows was evaluated in Experiment 3. The proportion of male embryos collected from the right uterine horns was significantly greater than from the left uterine horns. The sex ratio of embryos recovered from the uterine horns in this experiment was not different from parity. In an effort to determine the role of interovarian communications in the sex selection process, the sex ratio of embryos recovered from unilaterally ovariectomized superovulated beef heifers was investigated in Experiment 4. The sex ratios of embryos recovered from left- and right-ovary intact heifers were not significantly different. In Experiment 5, the sex ratio of IVP embryos was evaluated. The sex ratio of the IVP embryos was significantly lower than parity and was not different between ovary of origin. In addition, length of time in maturation was determined not to influence the sex ratio.
CHAPTER I

INTRODUCTION

Assisted reproductive technologies, including artificial insemination (AI) and embryo transfer (ET) have developed into powerful reproductive herd management techniques. Currently, AI alone, or in combination with an ET program, allows producers access to genetics they would normally be unable to obtain or afford to acquire. Bradford and Kennedy (1980) indicated that implementing an embryo transfer program in addition to AI would yield substantial genetic improvement. Most economically important genetic traits can be manipulated with relative ease (Land and Hill, 1975), while sex selection has, until recently, remained a matter of chance. Recent technological advances have allowed for the development of sex selection methods that can increase the probability of producing offspring of the desired sex. However, due to the relative high cost and limited efficiencies, the potential benefits of utilizing sex selecting techniques would most likely be realized in programs where the procedural cost would not outweigh the ability to disseminate the elite genetic potential (Seidel, 2003a). The development of simpler and relatively inexpensive sex selection techniques will increase the use of assisted reproductive technologies, such as AI and ET, in production animal agriculture.

Scientist and producers have long sought the ability to predetermine the sex of the conceptus. In fact, the Greeks posed this same question more than 2,500 years ago (cited by Gordon, 1979). The ability to predetermine the sex in farm animals would increase significantly the efficiency of livestock production. Foote and Miller (1971) postulated that an increase of up to 20% is possible in beef herds if the sex ratio of calves produced was increased to 85% male, while Cunningham (1975) hypothesized that dairy herds could increase milk production efficiency as much as 30% if female offspring could be selected at the time of insemination. The rate of genetic improvement can also be increased with the ability to predetermine the sex of the offspring (Seidel, 2003b). Van Vleck and Everett (1976) and Slanger and Anbil (1987) hypothesized that genetic improvement could be enhanced markedly with the ability to alter the normal sex ratio of calves produced.
Sex selection in the human population is also of interest for the prevention of X-linked diseases in families at risk. Approximately 6,600 heritable defects are known in humans and of these heritable defects, ~390 are known to be X-linked (Dobyns, 2006; Tsai and Gill, 2006). Generally, X-linked diseases are expressed by sons of carrier mothers who have inherited the X chromosome with the defective gene. The ability to effectively select for a female child prior to conception would eliminate the probability of conceiving affected males. Sex selection in this scenario represents a unique approach to disease prevention.

Researchers have attempted for decades to treat sperm in an effort to alter the sex ratio of the derived offspring. Ericsson et al. (1973) reported that fractions rich in Y-bearing sperm could be obtained in humans using a serum albumin gradient. These results were confirmed by other laboratories around the country (Dmowski et al., 1979; Ericsson et al., 1980; Quinlivan et al., 1982; Beernink et al., 1993; Bernstein, 1995). However, the ability to alter the sex ratio of offspring using a serum albumin layering technique is still a subject of contention, as many reports cite an inability to isolate fractions rich in Y-bearing sperm (Ross et al., 1975; Evans et al., 1975; Chen et al., 1997; Flaherty et al., 1997). Beal et al. (1984) attempted to isolate fractions rich in Y chromosome-bearing sperm in cattle using a similar method but found no difference in the proportion of X- and Y-bearing sperm. Currently, the most consistent and efficient method for selecting X- and Y-chromosome bearing sperm utilizes DNA staining and flow cytometric cell sorting (Johnson et al., 1989). Fractions with >90% purity of sexed bull sperm can now be consistently obtained. However, this technology is not without drawbacks and disadvantages. The rate at which sperm can be sexed, at this writing, is limited to ~15 x 10^6 cells per hour of each sex. In addition, DNA and cell membrane integrity may be compromised during the extended sorting procedure (Boe-Hansen et al., 2005; De Ambrogi et al., 2006).

While the limited number of sperm available is suitable for in vitro fertilization (IVF), conventional AI with a reduced number of sperm per insemination produces substantially lower pregnancy rates in dairy cattle (Linford, 1977). Consequently, alternative insemination procedures have been developed to take advantage of this
proven sperm-sexing technique using a reduced number of sperm (Seidel et al., 1997; Hylan et al., 2002).

Alterations in the offspring sex ratio in wild and free-ranging species have also been described in which maternal environmental conditions are implicated as a contributing factor. Numerous demographers and psychobiologists have noted marked changes in the secondary sex ratio. In the farm species, naturally occurring variations have been observed in swine (Parker and Bullard, 1913), sheep (Henning, 1939), goats (Sachdeva et al., 1973), cattle (Hohenboken et al., 1987) and horses (Qi, 1981). Researchers have hypothesized that the reasons behind these abnormalities include nutrition (Hoefs and Nowlan, 1994), social dominance rank (Clutton-Brock et al., 1984), coital rate prior to conception (Martin, 1995), season of birth (Nishida et al., 1974) and maternal steroid hormone concentrations at the time of fertilization (James, 1986).

While the data in these reports were obtained primarily through observational methods, experimentally derived evidence indicates that offspring sex may be partially influenced by the mother rather than solely determined as a circumstance of chance. Lane and Hyde (1973) reported that maternal stress could influence offspring sex and became the basis for the “Declining Maternal Conditions” hypothesis postulated by Trivers and Willard (1973). In addition, Clark and Galef (1990) observed that the uterine horn of gestation was partially responsible for offspring sex, while Clark et al. (1994) determined the ovary of origin, rather than the uterine horn, influenced offspring sex.

Maternal ability to control offspring sex due to environmental conditions, while controversial given the current understanding of the subject, offers insight into natural sex selection from an evolutionary point of view. Further investigations to determine the mechanisms underlying the variation observed with sex ratios are warranted in an effort to overcome the limited sorting rate and expenses incurred with flow-cytometrically sorted sperm.
CHAPTER II

REVIEW OF LITERATURE

Theories of Maternal Control of Offspring Sex

Theory of Declining Maternal Conditions

In many species, males compete more vigorously for mates than do the females of the same species. In these cases, male fitness may be more variable and indicative of future breeding success. As a result, male fitness is more strongly influenced by parental investment than is female fitness (Bateman, 1948; Trivers and Willard, 1973). For example, early growth is beneficial where breeding success of a male depends on body size. In this case, male fitness may be more strongly influenced by the amount of milk produced by the mother than is female fitness (Clutton-Brock et al., 1981).

Parental investment can have a greater effect on the breeding success of one sex compared with the other (Gabler and Voland, 1994). For example, in Red deer the sons of dominant mothers are more successful than their daughters, whereas, the daughters of subordinate mothers are more successful than their sons (Clutton-Brock et al., 1984). Similar results have also been observed in Rhesus macaques (Meikle et al., 1984). These effects occur partly because the breeding success of males is more variable and because early growth and juvenile survival are more strongly influenced by food shortage in males than in females (Clutton-Brock et al., 1985).

This situation leads to two possible predictions. First, where a given amount of parental investment has a greater effect on the fitness of males than on that of females, mothers would be expected to invest more heavily in individual sons than in individual daughters (Willson and Pianka, 1963; Reiter et al., 1978; Maynard-Smith, 1980). Studies in numerous mammalian species now indicate action on this prediction (Reiter et al., 1978; Clutton-Brock et al., 1981; Pickering, 1983; Kretzmann et al., 1993).

The second prediction is that mothers should bias the sex ratio of their offspring toward the sex whose production will have the greatest positive effect on their own fitness (Trivers and Willard, 1973; Okansen, 1981; Charnov, 1982; Frank, 1989). The advantage of producing male and female offspring to the mother will depend on three factors: (1) the relative fitness of the sons and daughters produced, (2) the costs to the
future reproductive success of the mother to produce and rear each sex and (3) any differences between the sexes of the offspring regarding cooperation or competition with the mother (McClure, 1981). A common prediction is that parents in superior condition, who are able to expend more energy or other resources, should produce sons, whereas, parents in inferior condition should produce daughters (Trivers and Willard, 1973). However, in some species, the phenotype of the mother can influence the breeding success of her daughters more than that of her sons. For example, in Rhesus monkeys, female offspring remain in their mother’s group and inherit her social rank and status (Simpson and Simpson, 1982). Trivers and Willard (1973) originally suggested that variation of the sex ratio might be accomplished by parental manipulation of postnatal mortality, suggesting that mothers should prematurely terminate investment in offspring that were unlikely to breed successfully or achieve their reproductive potential. Manipulation of the sex ratio before birth, however, would minimize wastage of reproductive effort and, where possible, would likely be favored (Maynard-Smith, 1980).

Predictions about the sex ratio are complicated due to the lack of knowledge of the comparative effects of parental investment on the fitness of sons and daughters, as well as the cost of rearing the two sexes (Clutton-Brock and Albon, 1982). Different types of maternal investment may be of greater importance to one sex than the other. For example, milk intake may affect the fitness of sons more than that of daughters, whereas social assistance after weaning may offer a greater advantage to daughters than to sons (Clutton-Brock et al., 1982).

In polytocous species, predictions about the sex ratio are further complicated by selection pressures favoring the most effective expression of reproductive potential (Williams, 1979; Burley, 1982; McGinley, 1984). For example, although mothers in poor condition might maximize their reproductive potential by producing a single daughter and those in marginally better condition might do so by producing a son, animals in superior condition might maximize their fitness by producing two daughters or a daughter and a son (Williams, 1979; Roche et al., 2006).

The hypothesis that mothers should vary the sex ratio of their progeny in relation to the profitability of the two sexes includes other adaptive predictions about variation in the sex ratio. However, arguments concerning the effects of the sex of the offspring on
the fitness of their siblings (Hamilton, 1967; Clark, 1978; Toro, 1982) or on the
subsequent reproductive success of their parents (Clutton-Brock et al., 1982; Malcolm
and Marten, 1982; Gowaty and Lennartz, 1985; Emlen et al., 1986) are generally
considered separate cases.

Societal Support Theories

Sibling Competition for Mates or Resources

If sons and daughters are equally costly to rear but siblings of one sex are more
likely to compete for mates than are those of the other sex, the sex ratio of progeny
should be biased toward the sex that will compete less intensely (Hamilton, 1967;
Maynard-Smith, 1980; Bulmer and Taylor, 1980; Taylor, 1981; Frank, 1985, 1989). The
original model by Hamilton (1967) was formulated to explain the strongly female-biased
sex ratios of many species of Hymenoptera. Due to the solitary nature of these species,
brothers compete to mate with their sisters. Under these conditions the production of a
large proportion of females not only reduces mating competition between siblings but is
likely to increase the mating success of the male siblings (Grafen, 1984). Changes in
the intensity of sibling competition or in the degree of inbreeding would favor changes in
the sex ratio (Colwell, 1981).

With competition between siblings for other resources, similar arguments can be
applied to differences in the sex ratio. In species in which sons disperse from their
rearing area while daughters share their mother’s home ranges, female siblings are
likely to compete for resources (Smith, 1968). Given this environment, the sex ratio of
the offspring should be male-biased (Clark, 1978). In species in which females live
matriarchal social units, changes in the intensity of competition between siblings,
changes in the sex ratio are often observed (Hoogland, 1981; van Schaik, 1983).

Competition between unrelated animals of the same sex can also favor variation
in the sex ratio. For example, in species in which one sex does not disperse, selection
may favor the production of the sex that disperses if the local population density has
reached the carrying capacity (McCullough, 1979). In species in which males disperse
following the maternal investment period, a positive relationship between population
density and sex ratio should exist (de Gayner, 1992).
Whenever generations overlap to a greater extent in one sex than the other, competition is likely to be greater in the shorter-lived sex in cohorts of above average size (Werren and Taylor, 1984). An excess of the longer-living sex would be predicted in years or seasons when large cohorts are produced.

**Parental Competition**

Competition between parent and offspring of a particular sex would also favor sex ratio variation. Where mature offspring of one sex adopt home ranges overlapping the ranges of their mother, their presence may reduce the mother’s subsequent reproductive success. In effect, this can be regarded as an additional form of parental investment, and should bias the sex ratio toward the sex that disperses more (Clutton-Brock et al., 1982).

Variation in the intensity of competition between parents and offspring of a particular sex may generate selection pressures that favor sex ratio variation. Whenever mature females share the range of their mother but sons disperse, daughters born early in a breeding life of a female will compete with her for a greater number of years than those born toward the end of her life, and selection would be expected to favor a negative relationship between the sex ratio and maternal age (Cockburn et al., 1985; McShea and Madison, 1994).

**Sibling Cooperation**

Cooperation between siblings can also affect the survival and reproductive success of the two sexes, and hence the sex ratio (Bulmer and Taylor, 1980; Taylor, 1981; Toro, 1982). When the offspring of one sex assist siblings of both sexes, a sex ratio biased toward the cooperating sex would be expected (Toro, 1982). An excess of the same sex would be expected wherever cooperation between siblings of one sex reduces the mother’s cost of rearing that sex. Since the benefits of cooperation are likely to change with the environment, both circumstances would favor variation in the sex ratio (Grafen, 1984).

When cooperation occurs between offspring of the same sex, alterations in the sex ratio are less predictable. Cooperation between male siblings that does not affect the breeding success of females would not affect the normal sex ratio for that species. For example, male lions cooperate with male siblings to dominate breeding access to
groups of females. A lion’s chance of obtaining a pride and that lion’s period of tenure increases with the number of resident males (Bygott et al., 1979). Under these conditions, females that produce all-male or all-female litters would be expected to have a higher fitness than those that produce litters with equal sex ratios. The average sex ratio would only be affected if females cannot alter the sex ratio within litters. Alternatively, in species where sisters assist each other but not brothers, a female biased sex ratio would be expected unless females can vary the sex ratio between successive litters.

Other types of interaction that influence the fitness of litter mates may also affect sex ratio. For example, rats reared in unisexual litters may adversely affect the sexual competence of males and females (Sharpe, 1975; Sharpe et al., 1973; Brian and Griffen, 1970).

**Parental Cooperation**

In certain species, resident offspring of one sex assist their parents’ attempts to reproduce. In these cases, the helping sex can be viewed as a partial repayment of the costs of their own production to their parents. Assuming that the initial costs of producing sons and daughters are the same, mothers would be expected to produce an excess of the cooperative sex under these conditions (Emlem et al., 1986). For example, in Red-cockaded woodpeckers and African Wild dogs, sons remain in their mother’s group to assist rearing subsequent litters. In both species, the sex ratio at birth shows a strong male bias (Gowaty and Lennartz, 1985; Malcolm and Marten, 1982).

In certain species of baboons and macaques, females assist relatives in competitive interactions. A female’s rank and breeding success can increase with the size of the maternal lineage to which she belongs (Wrangham, 1980). In these cases, mothers would be expected to bias their investment toward more daughters. However, increasing group size can depress the recruitment and breeding success of females (Silk, 1983). This effect would be expected to favor female-biased sex ratios during the establishment of groups or populations and male-biased sex ratios when the capacity of the local habitat has been reached (Armitage, 1991).
Juvenile Mortality During Parental Investment

Fisher (1930) postulated that a reduced viability among juveniles of one sex will favor the production of an initial preponderance of the less viable sex. This condition would be expected to reverse during the period of parental dependence until the sex ratio is biased against the less viable sex. Any tendency for one sex to die after the termination of parental investment would be offset by an increase in the average reproductive success of survivors (Leigh, 1970).

In certain sexually dimorphic mammals, juvenile males are less viable than females, especially when food is short or environmental conditions are harsh (MacArthur and Baillie, 1932; Clutton-Brock et al., 1982, 1985). The proportion of males produced would be expected to be inversely related to food availability (Skogland, 1996). However, a higher mortality of males before the end of parental investment would favor increased investment in sons. This would reduce any tendency to produce an excess of the less viable sex. In addition, selection pressures that favor the production of males at times when they are most likely to survive would reverse the tendency to produce an excess when resources are limited (MacArthur and Baillie, 1932).

Fluctuations in the Adult Sex Ratio

When the sex ratio of the adult population moves away from parity, mothers could temporarily increase their fitness by producing more offspring of the less numerous sex (Werren and Charnov, 1978; Charnov, 1982; Burley, 1982; Trivers and Hare, 1976). For example, after a population reduction that produces an adult sex ratio biased toward females, the production of males would be favored until differences in sex survival reappear within the adult population. Similarly, in certain rodent species, seasonal variation in the adult sex ratio would favor seasonal variation in the sex ratio at birth (McShea and Madison, 1994).

Inbreeding

Whenever males compete to mate with females to which they are related, two separate mechanisms are likely to alter the distribution of males and females. First, the resources and energy required to produce excess males negatively affects the mothers’ reproductive fitness and future breeding potential. Secondly, the contribution of producing daughters increases the average fitness of sons (Bulmer and Taylor, 1980;
Wilson and Cowell, 1981). In situations where brothers do not assist the breeding attempts of their sisters, the effects of inbreeding will not be distributed equally between the sexes and will favor an increase in the production of females (Bulmer and Taylor, 1980).

Many different factors may affect the comparative profitability of producing male and female offspring, and subsequently the sex ratio. Since no studies have yet been able to determine how parental characteristics affect both the fitness of male and female offspring and the comparative cost of raising the two sexes, it is not possible to predict the alteration of the sex ratio with certainty. Predictions are commonly post-hoc explanations in disguise. As there are few sex ratio trends for which it is impossible to construct an adaptive explanation, the fact that a trend can be explained or predicted is no guarantee that it is adaptive.

**Influences on Sex Ratio Variation**

**Litter Size**

Little definitive information is available regarding the effect of litter size on the sex ratio of offspring. In Norway rats (Blumberg et al., 1992) and guinea pigs (Peaker and Taylor, 1996), a negative correlation has been demonstrated between sex ratio and litter size in litters above average in size. Bacon and McClintock (1994) hypothesized that increased male mortality given limited uterine space accounted for the altered sex ratios.

The most abundant data for wild populations of larger mammals come from studies of New World deer, belonging to the genus *Odocoileus*. When data from the different populations were combined, the sex ratio among single births is higher than that among multiple births (Verme, 1983). However, there were apparent differences in age classes of the mothers. In one sample of mature, captive White-tailed deer, the sex ratio for single births was higher than for twins or triplets (Verne, 1969). The same study reported a lower sex ratio from single births in yearling does (30%). A study of a wild population of the same species also observed that the sex ratio of fetuses taken from mothers less than 2.5 years of age was significantly lower than that of fetuses taken from older females (de Gayner, 1992).

In three studies of sheep and one of cattle (Rasmussen, 1940; Napier and Mullany, 1974; Skjervold, 1979; Skjervold and James, 1979) the sex ratios for single
births were higher than among twins. However, two studies in sheep failed to show a similar pattern (Chapman and Lush, 1932; Johansson and Hansson, 1943). In all of these studies, deviations from parity were small. A study of Thoroughbred horses reported that the sex ratio among twins was significantly lower than the sex ratio of single births (Platt, 1978).

In humans, the sex ratio among dizygotic twins is higher than that for single births, while the sex ratio of monozygotic twins is lower (James 1975b, 1979). The sex ratio of same-sexed dizygotic twins is greater than that for opposite sexed twins (James, 1979).

Maternal Age

In Meadow voles (*Microtus pennsylvanicus*) older females produced more daughters and fewer sons in the fall of the year than did younger females (McShea and Madison, 1994). The authors hypothesized that the older females were closer to the end of their lifespan and were less likely to suffer from competition with daughters. A similar significant pattern has been observed in nutria (*Myocastor coypus*) (Gosling, 1998) and Golden hamsters (*Mesocricetus auratus*) (Huck et al., 1988), while in Yellow-bellied marmots, young females produced a significant excess of daughters (Armitage, 1991).

Among larger mammals, females usually start breeding before they have reached adult body weight, and toward the end of the lifespan, body condition declines as a result of repeated breeding. The sex ratio would be expected to be low among first breeders, to increase in the middle years of the lifespan, and to decline again in older animals. This prediction has been proven in various species, including sheep (Meadows, 1969; Caughley, 1971) and Red deer (Lowe, 1969). However, in some species these predictions are reversed, that is, first breeders are in superior body condition compared with middle-aged females. In these species, either an increase in breeding experience or a decline in reproductive value would encourage older mothers to invest more heavily in their offspring than do younger animals (Pianka and Parker, 1975). Additionally, in polytocous species, litter size normally varies with age, with both young and old mothers producing smaller litters than those of middle-aged females (Williams, 1979).
Only a limited number of studies of large mammals have reported any significant association between maternal age and the sex ratio. In a group of captive White-tailed deer does that produced single fawns, the sex ratio of fawns born to yearling females was significantly lower than that of fawns born to older females (30.0% vs. 66.7%, respectively) (Verne, 1969), and both samples differed significantly from parity. Results among twin-bearing mothers in this study did not differ from parity. In a similar investigation of fetal sex ratios in a wild population of White-tailed deer, de Gayner (1992) reported single births to young does had a lower sex ratio (32.0%) when compared with older females. However, when all data for Odocoileus were analyzed, the sex ratio of the offspring of yearling mothers (irrespective of litter size) was significantly higher than the sex ratios among the offspring of older mothers.

In two additional studies of ungulates where significant associations were found between the sex ratio and maternal age, older mothers produced higher sex ratios among their offspring than did younger mothers (Dapson et al., 1979; Flook, 1970). A higher sex ratio has also been reported among offspring from older Japanese macaque mothers (Noyes, 1982). However, numerous studies have investigated the relationship between maternal age and the sex ratio of offspring. No significant correlation has been reported in farmed Arctic foxes (Maciejowski, 1972), Roe deer (Borg, 1971), Mule deer (Robinette et al., 1957) and Barbary macaques (Paul and Thommen, 1984). Studies in humans indicate that the sex ratio declines with maternal age (James, 1974, 1976b, 1983). Additionally, nonsignificant trends in the same direction have also been reported (James and Rostron, 1985; Erickson, 1976; Garfinkel and Selvin, 1976; Imaizuma and Muraka, 1979).

Parity of the Mother

Although the theory postulated by Trivers and Willard (1973) proposed that the potential of a female for investment should decline with increasing parity, the relationship between parity and investment potential are complex. Most studies of parity are of mothers of similar age that have raised offspring the previous year and those that have not done so, with the latter generally being in superior body condition. Given the relatively small sample sizes, studies that investigate the sex ratio at birth to maternal
lifetime parity fail to demonstrate significant differences (Howard et al., 1955; Robinette et al., 1957; Clutton-Brock et al., 1982; Skjervold and James, 1979; Tomar et al., 1976)

In a sample of Mule deer, 2-year old females that had not mated the previous year produced significantly more male offspring than 2-year-old does that had mated as yearlings (Robinette et al., 1957). In Red deer, however, females that reared offspring the previous year produced significantly more male offspring compared with mothers that had not produced offspring (Miller, 1932). In sheep, the sex ratio at birth varied relative to the number of offspring reared the previous year, though the difference was small but significant (Skjervold, 1979). In Barbary macaques, mothers that failed to breed the previous season produced a significant excess of females (Paul and Thommen, 1984).

Studies of human sex ratios indicate that later children are significantly more likely to be female after the effects of maternal age have been taken into account (Novitzky and Kimball, 1958; Teitelbaum, 1972; Erickson, 1976; Garfinkel and Selvin, 1976; Imaizuma and Muraka, 1979; James, 1979; Astolfi and Tentoni, 1995), though the difference in probability is relatively small.

**Maternal Fecundity**

The relationship between the sex ratio and female fecundity has not been investigated in most mammalian species. Verme (1983) reported a negative association between the fecundity of different females and the sex ratio of their progeny in 29 different herds of White-tailed deer. In Rhesus macaques, a negative relationship between sex ratio and female fecundity has also been noted (Schino, 1999; Maestripieri, 2001). In humans, however, the sex ratio is positively correlated with the number of children (Repetto, 1972; James 1974, 1976).

**Nutrition**

Mothers maintained on a high plane of nutrition would be able to invest more heavily in their offspring than those on poor nutritional planes. In laboratory mice, females maintained on low-fat diets produced significantly female-biased sex ratios (24%), while those maintained on control diets produced litters with approximately equal sex ratios (Rivers and Crawford, 1974). In Golden hamsters, food restriction during pregnancy produced a significant reduction in the number of males per litter and a
decline in the sex ratio at birth (Labov et al., 1986). Subsequent experiments in which females were food restricted as juveniles showed reduced sex ratios produced as adults (Huck et al., 1986, 1987)

In the nutria (Myocastor coypu), maternal conditions can interact with age in its effect on the sex ratio. Gosling (1998) observed that young females sacrificed between 14 and 19 weeks of gestation carried significantly fewer female pups but the same numbers of males as mature females. According to the author, the difference occurred because young females in superior body condition aborted small, female-biased litters. The explanation proposed was that the production of small, female-biased litters did not permit females in good condition to realize their reproductive potential. Females in good body condition that aborted their litters conceived between 1.13 and 1.65 extra pups when they were mated again.

Among ungulates, White-tailed and Fallow deer maintained on a low level of nutrition produced a strongly male biased sex ratio compared with does maintained on a high nutritional level (Verme, 1969; Mulley, 1990). In goats, Sachedeva et al. (1973) reported offspring sex ratios increased as maternal nutrition decreased. However, in sheep, sex ratios rose as the weight of lambs born in the previous season decreased (Skjervold, 1979). These results are supported by negative relationships between sex ratio and habitat quality in ungulates.

In cattle, Skjervold and James (1979) observed that across herds the sex ratio increased with the milk yield of the mother. However, across individuals within herds high milk yields were associated with reduced sex ratios. Although significantly different, absolute differences in the sex ratio were not large. The authors hypothesized that the bias may have been due to higher mortality rates of males in utero. Further, it was argued that the contrary trends may have occurred because herds with low average milk yields are more likely to be nutritionally stressed than those herds with higher milk yields, and individual cows with high milk yields are more likely to be stressed than members of the same herd with lower milk yields.

Specific components of the diet may have an effect on the sex ratio at birth. Abel (1993) reported in rats, the sex ratio of offspring sired by males treated with alcohol was lower compared with control females. In the Chinese hamster, caffeine consumption
also produced female biased litters (Weathersbee et al., 1975). Cation concentrations in the diet may have an effect on the sex ratio at birth. In particular, diets high in sodium and potassium and low in calcium and magnesium reduced the sex ratio in rats (Cluzan, 1965; Bird and Contreras, 1986).

However, in cows (Stolkowski and Emmerich, 1971; Stolkowski and Lorrain, 1980), pigs (Bolet et al., 1982) and humans (Papa et al., 1983; Stolkowski and Choukroun, 1981), these diets increased the sex ratio of offspring at birth. Based on the results of Zolman and Valenta (1981) and Fitko and Szlezyngier (1994), James (1996) postulated that the increase in the sex ratio was due to increased gonadotrophin levels at the time of conception. In nonmammalian species, alteration in the dietary cation concentration has produced skewed sex ratios in birds (Riddle and Fischer, 1925), amphibians (King, 1912; Witschi, 1929), reptiles (Pieau, 1971) and insects (Suege, 1970, 1971).

Among humans, the sex ratio at birth increases with socioeconomic status, although the effect is not linear. The sex ratio of children born to families allocated to medium or high economic categories was significantly higher than the sex ratio of progenies allocated to the low economic category (Teitelbaum, 1970; Rostron and James, 1977).

Stress

Studies investigating nonnutritional stress have repeatedly demonstrated altered sex ratios in rats. Geiringer (1961), Lane and Hyde (1973), Moriya and Hiroshige (1978) and Gosling et al. (2001) reported stressed mothers produced significantly fewer sons than did unstressed mothers. Additionally, in two of the studies, the sex ratios produced by the experimental animals were significantly different from parity (Geiringer, 1961; Lane and Hyde, 1973). Similar results have also been reported in Chinese hamsters, with stressed mothers producing female-biased litters (Weathersbee et al., 1975).

In contrast, data on wild and captive primate populations suggest that stressful conditions may be associated with a relative increase in prenatal mortality in females, yielding a male-biased sex ratio at birth (van Schaik and van Noordwijk, 1983). The study compared infant sex ratios in wild and captive groups of primates, both of which commonly show male-biased sex ratios, with those produced by artificially provisioned
troops, which commonly produce female-biased sex ratios. This study was based on the supposition that animals in both wild and captive groups are living in more stressful conditions than those in provisioned troops. This alteration in the sex ratio parallels evidence that subordinate mothers produce more sons than daughters in some populations of monkeys (Silk, 1983).

In humans, nonnutritional stress in more difficult to define and is generally regarded as physiological rather than psychological. Mubarak and Mubarak (1996) observed that significantly more female children were born to families near high voltage electric lines compared with sex ratio of the general population.

Habitat Quality

The correlation between habitat quality of the mother and the sex ratio of the offspring is one of the earliest reported investigations in sex ratio variation (Combe, 1847, cited by Clutton-Brock et al., 1982). In sheep, ewes kept on poor pasture produced a higher sex ratio among their offspring than those maintained on improved pastures (Watson, 1982). Significant negative correlations have also been reported in other ungulates. In two studies including seven different Mule deer populations, the percentage of male fawns in hunter kills declined with increasing above ground forage availability (Brohn and Robb, 1955; Pederson and Harper, 1984). Fetal sex ratios declined with increasing food availability and maternal body weight among three different reindeer populations (Skogland, 1996). In both Red and Grey kangaroos, the sex ratio of young in the pouch declined as rainfall and available forage increased (Johnson and Jarman, 1983).

Population Demography

Numerous studies have reported that the sex ratios of offspring vary with aspects of population demography, including the juvenile sex ratio (Sharpe and Wyatt, 1974; Burley, 1980), the adult sex ratio (Maynard-Smith, 1980), population density (Emmerson, 1948), the phase of the population cycle (Nanmov et al., 1967; Budelov et al., 1978), the size of the maternal lineage of the mother (Armitage, 1991) and the extent of mortality among male and female juveniles in the previous season (Silk et al., 1981).

Studies of rodents have examined the effects of demography manipulating juvenile or adult sex ratios on the sex ratio in the next generation. In laboratory rats,
mothers reared in unisexual cross-fostered litters produced significantly fewer males than did those reared in bisexual litters (Sharpe and Wyatt, 1974). Mothers reared in unisexual litters also produced smaller litters but heavier pups (Sharpe et al., 1973).

Burley (1982) manipulated the sex ratio of litters of laboratory mice to produce three groups; mothers that reared only females, mothers that reared only males and control mothers that reared an approximately equal sex ratio of young. The study compared the sex ratio at birth of offspring born to those mothers of the three groups that produced between one and five litters during their lifespan. Overall, no significant differences were reported between the three categories. However, among mothers that produced three litters during their lifetime, those that reared only females produced significantly more male offspring. In addition, the sex ratios produced by experimental animals became progressively biased across successive litters.

In a wild population of woodchucks, Snyder (1962) removed adult females for two successive years. Compared with the control site, the fetal and juvenile sex ratios in the experimental populations produced a significant bias toward females. Similar results have also been reported in guppies (Geodakyan and Kosobutskii, 1969). However, Brown (1982) was unable to replicate the results in guppies obtained by Geodakyan and Kosobutskii (1969).

Demographic factors likely to affect competition or cooperation between relatives may influence the sex ratio of offspring. In Meadow voles (Microtus pennsylvanicus), the sex ratio of recruits is female-biased in the spring of the year and male biased in the fall, as a result of changes in the relative survival of the two sexes between birth and weaning (McShea and Madison, 1994). Differences between spring and autumn were associated with changes in the relative weights of pups. Females were heavier than males in the spring but lighter in the fall. The authors postulated that the seasonal change is adaptive because local population density and the probability of competition between related females increase during the year, favoring a female-biased sex ratio during the spring. Armitage (1991) proposed a similar argument to explain an increase in the percentage of female young produced by marmots belonging to small matrilines.

Competition between relatives provides a possible explanation of variation in pouch sex ratios among populations of Marsupial mice (Antechinus sp.) (Cockburn et al.,
In *Antechinus*, the majority of females reproduce only once in their lifetime and female offspring inherit their mothers' ranges, although in a few populations, some females breed more than once. Across populations, the pouch sex ratio with the parity of the mother. The hypothesis presented is that competition between mothers and daughters increases with the frequency of litters within seasons, favoring a reduction in the relative number of female offspring produced. This hypothesis is further supported by a tendency for Dusky antechinus females (*A. swainsonii*) to produce more males in the first litter than in subsequent litters. Strongly male biased sex ratios among pouch young have also been reported in other marsupials, whose daughters are likely to compete with their mothers (Poole, 1985).

In social mammals, the presence of mature daughters may enhance their mother’s reproductive success. In Richardson’s Ground squirrels, Michener (1980) observed that the two matrilines that were the most successful in generating recruits to the breeding population produced a higher percentage of female young than the less successful matrilines, although the cause of this association remains unclear.

Data from White-tailed deer indicate that the sex ratios among juveniles may increase with population density (McCullough, 1979). In another study of White-tailed deer, Richter and Labisky (1985) reported that fetal sex ratios increased with hunting pressure, which reduced the proportion of adult females. However, in Elephant seals, Le Boeuf and Briggs (1977) revealed that the sex ratio of juveniles increased along with population density. Though significantly different, the deviations from parity were small. In contrast, no significant associations were detected between matriline size or population density and birth sex ratio in wolves (Mech, 1995). In captive Bonnet macaques, an association between sex ratio at birth and the survival of male and female offspring in the previous year was suggested (Silk et al., 1981). However, no statistically significant correlations were reported.

**Social Dominance Order**

The relationship between maternal dominance rank and the sex ratio of offspring is the subject of much controversy. In wild populations of Yellow baboons, Hausfater et al. (1982) observed that high-ranking mothers produced fewer sons (34.5%) than were produced by subordinate mothers (68.2%). These data were later confirmed by Altmann
et al. (1985) and Samuels et al. (1987) in different populations of Yellow baboons. Simpson and Simpson (1982), investigating two separate Rhesus monkey troops, reported that high-ranking females from both groups produced a significantly lower proportion of sons (25% and 31%) than did subordinate females (68% and 61%). In all of these studies, the sex ratio of progeny born to dominant mothers was significantly lower than parity.

Other studies of nonhuman primates have failed to find parallel effects. The effect of maternal dominance on the sex ratio was not found to be significant in another colony of Rhesus monkeys (Small and Smith, 1985) or in a colony of Japanese macaques (Fedigan et al., 1986). Additionally, Silk (1990) failed to find a significant correlation in Bonnet macaques for low-ranking mothers to produce a male-biased sex ratio (63%), although dominant females produced offspring with sex ratios approaching parity (52%). In an introduced population of Rhesus macaques, Gomendio (1989) observed that middle-ranking lineages produced a lower sex ratio than either high-ranking or low-ranking matrilines. However, the fecundity of the middle-ranking females was higher than that of the low-ranking and high-ranking mothers. In addition, the body condition of the middle-ranking females was possibly superior.

Conversely, additional studies in non-human primates have reported effects contradictory to those reported by Hausfater et al. (1982), Altmann et al. (1985), Samuels et al. (1987) and Simpson and Simpson (1982). In an introduced population of Rhesus macaques, Meikle et al. (1984) observed significantly more male offspring born into high-ranking than into low-ranking matrilines. In addition, the sex ratio increased with the relative rank of the troop to which the mother was a member. Similar results were also reported in Rhesus macaques by Breuggeman (1973) and Sade et al. (1988), as well as Barbary macaques (Paul and Thommen, 1984) and pigtailed macaques (Sackett et al., 2002).

In Red deer, the sex ratio of the offspring has been reported to increase with maternal rank (Veiberg, 2004). The sex ratio produced by dominant mothers (61%) differed significantly from parity, while the sex ratio produced by subordinate mothers (47%) did not differ from parity. However, no difference in the sex ratio at birth between high-ranking and low-ranking Red deer mothers has also been reported (Lincoln, 1970).
In humans, dominance, as a personality trait, is defined as influential, ascendant, prevailing, or authoritative, and must be discriminated from aggressive, hostile, angry, violent, domineering and dictatorial (Sadalla et al., 1988). Fiske (1971) defined the core of dominance as “acting overtly so as to change the views or actions of another”. The maternal dominance hypothesis, first postulated by Moss (1967), states that women who are more dominant in personality compared with women in their cultural group, are more likely to conceive and bear sons. Various studies have reported results consistent with this hypothesis (Grant, 1990, 1994, 1996; Singh and Zambarano, 1997; Marleau and Saucier, 2000; Bogaert, 2001), with dominant women, as determined by personality tests, producing significantly more male offspring compared with women classified as subordinate. James (1985) proposed that women classified as dominant had higher levels of estrogen and testosterone at the time of conception which would influence the sex of the child.

Few studies of other mammals have been able to investigate relationships between maternal social rank and sex ratios. In Golden hamsters, the sex ratio of offspring from dominant females was significantly higher than the sex ratio produced by subordinate females (Huck et al., 1988). However, these results should be interpreted with caution as dominance was established between individuals during periods of restricted food intake.

Climatic Variation

Sex ratios at birth have been suggested as being affected by climatic factors. In Deer mice, exposure to higher than average temperatures during early pregnancy in the spring are associated with significant increases in litter size and in the number of female pups, but not male pups, born per litter (Myers et al., 1985). The authors suggested the altered sex ratio was a result of reduced wastage of female embryos. In contrast, higher than normal average temperatures in the autumn were reported to be associated with a significant reduction in litter size and a significant decrease in the number of male pups born per litter. Subsequent experiments indicated that high temperatures at or just prior to implantation resulted in increased mortality of female embryos, but after implantation resulted in an increased mortality of male embryos (Myers and Master, 1986; Millar and Millar, 1989).
In Mule deer, a negative relationship between the sex ratios at birth and the severity of the winter environment has been reported (Kruuk, 1999; Coulson, 2002). However, in Roe deer and Red deer the relationship between winter severity and sex ratios has been reported as positive (Borg, 1971; Guinness et al., 1978).

Season of Birth

Nishida et al. (1977) compared the sex ratio of piglets born during the various seasons of the year. Significantly more males were born during the spring of the year compared with the sex of offspring born during the summer, fall and winter. In *Bos tarus* cattle, more male calves were reported born in the spring and winter months when compared with calves born during the summer and fall months (Morgan and Davis, 1938; Maramatsu and Kawanishi, 1975; Tomar et al., 1976). In contrast, a study of *Bos indicus* cattle in West Africa reported that sex ratios at birth were higher among calves born between September and December, when food was relatively abundant, than among animals produced during the rest of the year (Denis, 1978). Although the difference was interpreted as being an effect of the month on the sex ratio at conception, an alternative explanation is that the difference was caused by a reduced survival of male fetuses during the latter months of the dry season (Denis, 1978).

Timing of Insemination

Studies in pigs and cattle have compared sex ratios resulting from artificial insemination compared with natural mating (Nishida et al., 1977; Maramatsu and Kawanishi, 1975). While no significant differences were found in relation to type of insemination method, variation in the timing of insemination, relative to ovulation, was determined to be related to the sex ratio. Hammond (1934) reported that in rabbits fewer male offspring were produced when does were mated early. In addition, Hedricks and Mcclintock (1990) reported in Norway rats an increased sex ratio with matings early in estrus compared with late-estrus matings. Conversely, in White-tailed deer, matings shortly after the onset of estrus produced female biased sex ratios, while those occurring 36 hours after the onset of estrus produced male biased sex ratios (Verme and Ozoga, 1981). Ovulation in this species occurs 12 to 14 hours after the end of estrus. In sheep, Gutierrez-Adan et al. (1999) produced male-biased sex ratios when inseminations were
conducted after ovulation compared with the female-biased sex ratios obtained with inseminations prior to ovulation.

In cattle, however, the effect of insemination timing remains controversial. Riddle (1919), Wehner et al. (1997), Wells (1980) and Martinez et al. (2004) have reported altered sex ratios relative to breeding time similar to those observed in White-tailed deer. In contrast to these reports, Foote (1977), Rorie et al. (1999), Ballinger (1970) and Roelofs et al. (2006a, 2006b) determined that sex ratios were not significantly different from parity. Ostrowski (1988) reported significantly more female calves in heifers that were estrus synchronized with PGF$_{2\alpha}$ and GnRH compared with heifers synchronized with PGF$_{2\alpha}$. The author hypothesized that GnRH allowed pasture breeding to occur earlier, relative to the time of ovulation, than in the PGF$_{2\alpha}$ only group. However, James (1989) argued the results obtained by Ostrowski (1988) were due to maternal hormone levels at the time of insemination and not influenced by the timing of insemination.

In humans, more males are born as a consequence of natural insemination three or more days before, or one or more days after ovulation (Guerrero, 1970, 1974; Harlap 1979; James, 1971, 1983). James (1980, 1983) postulated that this trend could occur because maternal gonadotropin levels peak around the time of ovulation, thereby “activating” the female tract and preventing the separation of X-bearing and Y-bearing sperm through gravitation as proposed by Roberts (1978). The sex ratio of human infants born after ovulation has been induced by clomiphene is unusually low (43.6%) compared with the population average (51.4%) (James, 1980). However, one difficulty with this explanation is that artificial insemination is associated with an increase in the sex ratio of children born as a result of insemination close to the day of ovulation (Guerrero, 1974). In contrast, Wilcox et al. (1995) reported that the timing of sexual intercourse relative to the time of ovulation produced similar sex ratios at birth between cycles.

Variation in the timing of insemination has been suggested as a likely cause of different correlations between the sex ratio and other variables, including maternal rank (Simpson and Simpson, 1982) as well as the proportion of adult males in the population (Richter and Labisky, 1985).
Ovary of Origin

Asymmetric distribution of the sexes within the uterus of pregnant polytocous mammals has been described in numerous species. YoungLai (1981) reported more male fetuses in the left uterine horn and more females in the right uterine horn in the rabbit, while Bruce and Norman (1975) and Herbert and Bruce (1980) failed to find a difference in the uterine horn sex ratio in the rat. In the mouse, however, conflicting results have been reported. Endo et al. (1987) and Sakai and Endo (1987) found more male mice fetuses in the right horn and females in the left horn, while Clark et al. (1991) were unable to detect any sexual segregation within the uterine horns in the mouse. Clark and Galef (1990, 1995) described an excess of males on the right and females on the left in the Mongolian gerbil. In an effort to determine the source of the uterine horn sex ratio variation, Clark et al. (1994) surgically translocated the left and right ovaries within Mongolian gerbil females. These authors reported an inversion of the sex ratio within the uterine horns of the treated animals, with more females on the right and males on the left and hypothesized that “ovarian substances” were responsible for the sexual segregation rather than the uterine environment.

In the pig, James (1982) examined the sexes of piglets within the uterine horns and noted that the sexes were not associated with the side of the uterine horn in which they were gestated. However, transuterine migration in which embryos are distributed between the uterine horns has been reported in the sow (Warwick, 1926; Dhindsa et al., 1967) and may mask the effect of side of gestation and ovary of origin in the pig.

In humans, reports have failed to illuminate any sexual segregation in embryos or fetuses from left and right ovary derived oocytes. However, James (2001), based on the work of Fuduka et al. (2000) and Clark et al. (1994), hypothesized that higher concentrations of estradiol and testosterone should be associated with the right ovary and should produce an excess of males. However, the notion that the ovary of origin influencing the sex of the resulting offspring has been ridiculed (Cohen and Stewart, 1998), regardless of the species in question.

Directional Orientation

First appearing in the popular press, Dahl (1974) reported that in cattle, directional orientation at the time of breeding could influence the sex of the calf.
According to the author, facing the female into the sun at the time of insemination (timed mating or artificial insemination) would produce an excess of females. The premise behind this sex selection technique is that the gravitational pull of the sun provides a competitive advantage to sperm that carry an X chromosome compared with Y-chromosome bearing sperm. Bradley (1976) later confirmed the hypothesis postulated by Dahl (1974) and reported a sex ratio of 83.3% female in animals mated facing the sun compared with a sex ratio of 39.1% female in control animals mated in random directions or facing north. Statistical analysis performed by Wishart (1977) on the data reported by Dahl (1974) and Bradley (1976) indicated that significantly more (P<0.001) female calves were produced when inseminations were conducted on sun- and south-facing cows compared with random or north-facing cows. Though the author concluded that the experimental methods and data collection presented by Dahl (1974) and Bradley (1976) were less than ideal, in a properly designed experiment, a small sample of as few as 30 cows would be required to demonstrate a significant difference in the sex ratio (P<0.05).

**Sexed Semen**

Though the mechanisms under which sex ratio is controlled in nature remains unknown, researchers have attempted to treat sperm in an effort to predetermine the sex of the resulting offspring. Kovalev (1980) treated sperm to a variety of magnetic field intensities in an attempt to alter the sex ratio of calves, while Hafs and Boyd (1974) employed electrophoresis in an attempt to isolate rich fractions. Electrostatic fields (Soeradia and Tadjudin, 1986) were reported to have no effect on the ability to differentiate sperm into fractions rich in X- or Y-bearing sperm. Luderer et al. (1982) attempted to isolate sperm fractions on Newtonian gels, while Iwasaki et al. (1988) attempted enrichment of X-chromosome bearing sperm using Percoll density gradient centrifugation. Seminal pH was altered in a study by Muehleis and Long (1976) in an effort to alter the sex ratio; however, no significant difference was noted between control animals and treatment animals. Bedford and Bibeau (1967) and Courot and Esnault (1973) concluded that sperm sedimentation failed to influence the sex ratio in rabbits and cattle, respectively. Gravitational separation has also been proposed as a possible method of sperm separation (Roberts, 1972).
While the vast majority of these treatments have resulted in failure to isolate fractions rich in either X or Y chromosome-bearing sperm, occasionally limited success is reported. Blecher et al. (1999) reported an immunological sperm sexing method based on Ohno’s law. Ohno (1967) proposed that a large proportion of genes on the mammalian X chromosome are highly evolutionary conserved; an X-linked gene in any mammalian species would predict the existence of a homologue in other mammalian species. Using rabbits, Blecher et al. (1999) reported the production of polyclonal antibodies specific to cell surface proteins unique to X- and Y-chromosome bearing bull sperm, respectively.

Utilizing the antibodies prior to fertilization, treated sperm produced viable embryos with a sex ratio of 92.0% male as determined by polymerase chain reaction (PCR). In contrast, Howes et al. (1997) was unable to detect sex-specific surface proteins using various techniques, though nonsurface X-chromosome specific proteins were detected. Numerous studies have also reported an inability to detect sex-specific antigens (Sills et al., 1998; Hendriksen et al., 1993; Bradley et al., 1987; Hancock and Faruki, 1986; McArthur et al., 1981; Bennett and Boyd, 1973) though the existence of sex-specific surface proteins on mammalian sperm have been proposed as theoretically possible (Hendriksen, 1999).

The only two primary methods of sperm separation that have repeatedly been reported to isolate fractions rich in X and/or Y chromosome-bearing sperm are serum albumin gradient swim-up and flow cytometric sorting. However, the ability of the serum albumin gradient method to isolate Y-chromosome rich fractions and alter the sex ratio of offspring is still a subject of controversy.

**Serum Albumin Layering**

Ericsson et al. (1973) first reported that when human sperm were layered on columns of liquid albumin, ~85% of the resulting sperm migrating to the lowest portions of the columns bore a Y chromosome. The results of this study soon became controversial when Ross et al. (1975) and Evans et al. (1975) reported that serum albumin layering did not isolate fractions rich in Y-bearing sperm. Later reports from numerous IVF laboratories around the country (Dmowski et al., 1979; Burstein and Schenker, 1985; Beernink and Ericsson, 1982; Perrone and Testart, 1985; Quinlivan et
al., 1982; Beernink et al., 1993; Bernstein, 1995) have confirmed the findings of Ericsson et al. (1973). Ueda and Yanagimachi (1987), David et al. (1977), Reubinoff and Schenker (1996), Han et al. (1993) and Brandriff et al. (1986) have also reported that albumin layering alters the sex ratio of offspring ranging from 73 to 90% male.

Ericsson (1989, 1994) contended that technician error in procedure implementation is responsible for the contradictory results reported from other laboratories. Other possible reasons for the contradictory results were discussed by Levin (1987). Reasons included varying concentrations of serum albumin in the layers and removing more than 3% of the total sperm number from the sample. Meistrich (1982) reported that when more than 3% of total sperm number was allowed to pass through the albumin gradients, an altered sex ratio was not detected.

Serum albumin layering of sperm has again met with some success in certain farm species. Altered sex ratios have been reported in sheep (White et al., 1984a). However, in swine (Dixon et al., 1980) and horses (Goodeaux and Krieder, 1978), treatment of sperm with the Ericsson method has failed to produce altered sex ratios. Furthermore, in cattle, reports of successful sex ratio alterations have been conflicting. Ericsson et al. (1980) reported a statistically significant difference in the sex ratio of offspring from semen that was treated with a BSA gradient (three bulls). However, when offspring from a fourth bull was included, the difference in sex ratio was not significantly different.

Foote (1985) also reported an altered sex ratio in crossbred beef heifers inseminated with treated sperm. Reports of unaltered sex ratios in cattle, following serum albumin separated sperm, have also been published (White et al., 1984b). Beal et al. (1984) reported the proportion of male offspring with separated semen was ~45% male, while semen from unseparated controls yielded a sex ratio of 54% male offspring. These results agree with the findings of Ferguson et al. (1976) in which an altered sex ratio was not detected in cattle following treatment of sperm.

Amann (1989) postulated that serum albumin layering may not alter the proportion of Y-bearing sperm, but may alter the ability of Y-bearing sperm to fertilize the ovum. Although White et al. (1984b) reported that sperm obtained in this matter had fewer primary abnormalities and a greater motility, data obtained by flow cytometry
offers evidence that populations of bovine sperm isolated by the Ericsson procedure are not enriched for Y-bearing sperm (Garner et al., 1983; Pinkle et al., 1983; Beal et al., 1984; Johnson, 1988; Upreti et al., 1988).

Flow Cytometric Sorting

The development of modern flow cytometric cell sorting is credited to Fulwyler (1965) and Kamentsky and Melamed (1967). Flow sorting systems were commercialized in the 1970s and developed rapidly in conjunction with the computer revolution in the 1980s and continued in the 1990s. Although the primary application has been in medical research and diagnosis with respect to erythrocytes, flow cytometry is an effective tool for many types of cell suspensions. Sperm in suspension are readily adapted to flow analysis and sorting. The usefulness of flow cytometry is illustrated by the ability to measure the relative DNA content of individual sperm at a relatively rapid rate (Johnson, 1992).

Sorting of sperm into X and Y populations using flow cytometry is based on a simple design, though it is immensely complex in its implementation (Johnson, 1992). Briefly, sperm are stained with Hoechst 33342, which binds to the DNA in an amount proportional to the amount of DNA present. The stained sperm in suspension are then forced under pressure through an orienting nozzle, which intersects an argon laser. When excited by the laser, Hoechst 33342 fluoresces with intensity proportional to the amount of stain present, and is relative to the amount of DNA present in the individual sperm cells. Two photomultiplier tubes at 0° and 90°, relative to the nozzle stream, detect this fluorescence. The relative DNA content is calculated by computer and is determined to be X-bearing, Y-bearing or unusable portions. The droplet containing the evaluated sperm is electrically charged immediately after analysis based on computer determination of characteristics. As the droplets fall, deflection plates sort the charged particles into the respective populations (Johnson, 1992).

Gledhill et al. (1976) pioneered the use of flow cytometry for evaluating sperm DNA content to use as an indicator of mutagenic events. Mammalian sperm, with the exception of the Creeping vole (Microtus oregoni) (Ohno et al., 1963; Johnson and Clarke, 1990) all carry an X or Y chromosome and nuclear DNA content differs in X and Y chromosome-bearing sperm of all mammals evaluated (Ohno, 1967). Although the
DNA content of the autosomes does not differ between the X- and Y-bearing sperm, the heterologous DNA in the sex chromosomes do differ. This difference in DNA mass between the X and Y chromosome of sperm is the only established and measurable differential parameter. However, a difference of at least 3% in DNA mass between X- and Y-bearing sperm is necessary for accurate analysis and separation (Johnson and Pinkle, 1986; Johnson et al., 1987a).

The first flow sorting of sperm for purposes of isolating X from Y chromosome-bearing sperm was reported by Pinkel et al. (1982) using sperm from the Creeping vole (Microtus oregoni). The Creeping vole was selected because of the large difference (9.1%) in DNA content between X- and Y-bearing sperm. However, chinchilla sperm has now become the standard for equipment regulation and adjustment because this species also possesses a large difference in mass (7.5%) between X and Y chromosome-bearing sperm and the chinchilla is easier to maintain in colonies (Johnson et al., 1987b).

Originally, sperm nuclei, instead of intact sperm, were flow sorted because of the ease of cell orientation in respect to the laser beam (Johnson et al., 1987b). Tails were removed from the sperm with sonication. Sperm nuclei from the bull, boar, ram, chinchilla, Creeping vole (Johnson et al., 1987b; Johnson and Clarke, 1988), rabbit (Johnson et al., 1989) and human (Johnson et al., 1993) have been sorted into two populations using the flow cytometer. The initial viability testing of the sorted sperm nuclei was determined through microinjection of sperm heads into hamster oocytes (Johnson and Clarke, 1988) and culturing the oocytes through the pronuclear stage. Sorted sperm nuclei have also been microinjected into sheep oocytes and subsequently cultured to the 16-cell stage (Clarke et al., 1988).

Sorting of intact, viable sperm differs from sperm nuclei separation in sperm preparation and cytometer modification. A beveled nozzle (76 mm) is used to correctly orient the sperm for proper alignment with the laser beam for sorting (Johnson and Pinkle, 1986). Viable sperm preparation and staining differs in the absence of sonication and the added step of incubation at 35°C, to facilitate the rapid stain penetration into the sperm (Johnson et al., 1987a; Johnson et al., 1989; Johnson, 1991). There has been one other report of sorting viable sperm. Morrell et al. (1988) used an unmodified
commercial flow cytometer to sort Hoechst 33342 treated bull and rabbit sperm. However, reanalysis for proportions of X- and Y-bearing sperm could not be performed and fertility results showed some alteration of the sex ratio of the offspring produced. However, the sex ratio of the calves was not significantly altered from the expected ratio of 50%.

Fertility results following IVF with sorted sperm have yielded positive results with certain laboratory and farm species. Rath et al. (1993) reported that the sex ratio of piglets born following transfer of IVF-derived embryos was significantly altered. This pattern in swine was later confirmed by Rath et al. (1996, 1997). Cran et al. (1993) reported the birth of the IVF first calves using flow cytometrically sorted semen. While the sex ratio of the calves born (n=4) was not significantly altered, the sex ratio of sexed embryos not transferred was almost identical to the proportions of X- and Y-bearing sperm present in the semen. In a larger field study using IVF embryos, Cran et al. (1995) reported significantly altered sex ratios in dairy calves. However, Johnson et al. (1994) postulated that for sexed semen to make a dramatic impact on the cattle industry, an increase in the rate of sperm sorting, one that would allow females to be inseminated artificially, would be needed. Catt et al. (1996) reported the birth of a male lamb following intracytoplasmic injection of a Y-sorted sperm. Because only a single male lamb was born, this study did not reveal an altered sex ratio in offspring. However, reanalysis of sorted sperm indicated a sharply skewed ratio (92%) of X- and Y-bearing sperm. Offspring derived from insemination with sexed sperm do not display any greater genetic abnormalities that normally observed in unsexed sperm samples (McNutt and Johnson, 1996).

The sex ratio of offspring following artificial insemination with sexed semen has also yielded positive results. Johnson et al. (1989) reported the birth of the first offspring using flow cytometrically sorted sperm in rabbits. The sex ratio of the 37 offspring produced was similar to the sex ratio of the X- and Y-sorted sperm used for fertilization and was significantly altered from the expected sex ratio of 50% (6% and 81% male, respectively).

Although sperm of all species can be sorted with high purity, achieving acceptable pregnancy rates using the low numbers of sperm needed for commercial
application remains a major challenge in swine. Johnson (1991) has reported altered sex ratios in sows surgically inseminated with sexed sperm. However, Johnson (1997) indicated that AI of sows and gilts with sexed sperm, using standard nonsurgical methods, would require further advances in sorting technology, due to the large number of sperm required for insemination compared with other farm animal species. Krueger et al. (1999) and Krueger and Rath (2000) demonstrated that $50 \times 10^6$ sperm surgically deposited in close proximity to the utero-tubual junction were sufficient to obtain fertilization. Currently, nonsurgical deep uterine insemination utilizing specially designed disposable catheters with a reduced number of sex-sorted sperm is possible (Martinez et al., 2001, 2002; Grossfield et al., 2005). However, Rath et al. (2003) reported a reduced pregnancy rate and reduced litter size when employing this insemination method with sex-sorted sperm.

In cattle, Seidel et al. (1996) reported the birth of calves following AI of cows with an ultra-low number of sexed sperm. The results of this study were later confirmed by Seidel et al. (1997, 1998, 1999). In all of these studies, the sex ratio of calves born from females inseminated with X- or Y-bearing sperm was significantly altered from the expected 50% sex ratio (~10% and ~90% male, respectively). The number of offspring produced utilizing sperm sexed by flow-cytometric sorting is estimated to be greater than 30,000, with the majority of births occurring in cattle (Johnson et al., 2005).

While this technology has proven to be an effective method for altering the sex ratio of sperm and the subsequently derived offspring, limitations in the number of sperm cells that can be sexed in a given amount of time remains a major hurdle. Rens et al. (1998) reported the development of a novel nozzle that improves the efficiency of sexing sperm and has led to an increase in the number of cells that can be processed. Currently, sperm can be produced at a sorting rate of $15 \times 10^6$ sperm cells per hour per sex (Johnson et al., 2005). In addition, a recent surge in the commercial production of IVF-derived cattle embryos is at least partially due to the use of flow-cytometrically, sex-sorted sperm (Wheeler et al., 2006). This ability to skew the sex ratio in the desired direction prior to fertilization and produce genetically superior offspring justifies the added expenses due to sorting cost and production inefficiencies.
CHAPTER III

DISTRIBUTION OF SEXES WITHIN THE LEFT AND RIGHT UTERINE HORNS OF BEEF CATTLE

Introduction

Asymmetric distribution of the sexes within the uterus of pregnant mammals has been described in numerous laboratory species. An excess of female fetuses has been reported in the right uterine horn of the rabbit (YoungLai, 1981), while in the mouse (Endo et al., 1987) and Mongolian gerbil (Clark and Galef, 1991) an excess of male fetuses in the right horn has been observed. In contrast, Clark et al. (1991) failed to detect any sexual segregation within the uterine horns in the mouse, while Bruce and Norman (1975) and Herbert and Bruce (1980) also failed to find a statistical difference in the sex ratio between the left and right uterine horns in the rat.

The partial segregation of sexes observed in the uterus of the gerbil, rabbit and mouse suggest some consistent lateral asymmetry either between the left and right uterine horns or the left and right ovaries in these species. The literature provides numerous examples of lateral asymmetry of both mammalian ovaries and uterine horns (Pearson, 1949; Wimsatt, 1975, 1979; Baird and Birney, 1985).

In rats, the left uterine horn contains fewer implantation sites than the right horn (Buchanan, 1974). A larger number of embryos are gestated in the right uterine horn in mice (Wiebold and Becker, 1987), and in hamsters, a greater number of sperm are present in the right uterine horn after mating (O and Chow, 1987).

The right ovary in humans produces more ovulations (Fukuda et al., 2000) and is more prone to develop tumors (Willis, 1967), while in shrews, the left ovary contains a greater number of follicles (Mohanty and Chainy, 1992) compared with the right ovary. In rats, the right ovary is larger than the left (Mittwoch and Kirk, 1975) and in hamsters, mice and rats the right ovary contains more corpora lutea (Buchanan, 1979; Fritzsche et al., 2000; Long et al., 1991). In pigs (Hunter et al., 1985), humans (Schwartz et al., 1980; van Niekirk and Retirf, 1981), guinea pigs (Jaffe and Papanicolaou, 1927) and hamsters (Kirkman, 1958) testes or ovotestes in hermaphrodites occur predominantly on the right side. In contrast, however, testes or ovotestes occur predominantly on the left in the mouse (Ward et al., 1987).
In farm species, Arthur (1958) described ovarian activity in the mare and noted a greater proportion of corpora lutea (CLs) present on the left ovary compared with the right ovary. Henning (1939) and Casida et al. (1966) reported that the right ovary in sheep produces more corpora lutea than the left ovary. Similarly, in the goat, Taneja (1959) and Basu et al. (1961) discovered that the right ovary was more active than the left ovary, having a greater number of large follicles. Likewise, Lyngset (1968) found that in the goat, CLs were predominantly found on the right ovary compared with the left ovary in both single and multiple ovulations. Reese and Turner (1938) detected the corpus luteum (CL) in the right ovary more often than in the left ovary of heifers, while Rajakoski (1959) observed a significantly higher number of follicles ≥5 mm in the right ovary than in the left in mature cows.

James (1982) examined the sexes of piglets within the uterine horns of sows and noted that the sexes were not associated with the side of the uterine horn in which they were gestated. However, transuterine migration in which embryos disperse within and between the uterine horns has been previously described in the pig (Warwick, 1926; Dhindsa et al., 1967).

In cattle, no information is available regarding the sex ratio of the offspring in relation to the horn of gestation. The specific objective of this study was, therefore, to determine the sex ratio of fetuses and calves gestated in the left and right uterine horns in cows.

**Materials and Methods**

**Experiment 3.1**

**Collection of Reproductive Tracts**

Reproductive tracts from pregnant cows and heifers collected at a local abattoir were investigated in this experiment. Complete reproductive tracts, including the ovaries, oviducts, uterus, cervix, vagina and external genitalia, were requested from all pregnant females processed on a particular day, regardless of age, breed or gestational duration. The gravid tracts for this experiment were collected primarily from mixed-breed beef cows and dairy cows, consisting of Holstein and Jersey breeds. After removal from the animal, the tracts were immediately placed in a plastic storage container and placed in a refrigerated cooler (~4°C) to await processing.
Once the technician arrived at the abattoir, the individual tracts were recovered from the holding cooler and placed into large plastic bags. The pregnant tracts were transported to the laboratory at 22°C to 25°C within 8 to 10 hours after collection at the abattoir.

**Horn of Gestation Verification**

Upon arrival to the laboratory, tracts were removed from the plastic bag and placed onto a table for examination to determine the uterine horn of gestation. Proper orientation of the tracts was assured based on the greater curvature of the horns and the correct alignment of the external genitalia. Gravid reproductive tracts in which the horn of gestation could not be determined due to advanced gestational length or incomplete tracts were not used in this study.

**Sex Determination**

After the horn of gestation was recorded, an incision was made along the length of the uterine horn and the fetus was dissected from the fetal membranes and removed from the uterus. The sex of the fetus was determined by visual inspection of the external genitalia. Any fetus in which the sex could not be determined due to short gestational length was not used in this study.

**Experiment 3.2**

**Experimental Animals**

In this study, a group of artificially inseminated, crossbred beef heifers were evaluated over a 2-year period to determine the effect of horn of gestation on the sex ratio of calves. These animals were various crosses of Angus, Brangus, Brahman, Hereford and Simmental breeds. All experimental females were in excellent health and good body condition at the onset of treatment. Animal age ranged from 14 to 16 months with a mean (±SEM) of 14.8±0.3. Body weights ranged from 272 to 375 kg with a mean (±SEM) of 320.6±54.9 kg. These randomly cycling nulliparous females were selected from a large experimental herd maintained at Dean Lee Research Station, located in Alexandria, Louisiana.

During this study, females were maintained in large pastures established with bermudagrass (*Cynodon dactylon*) and overseeded with ryegrass (*Lolium multiflorum*). Experimental animals were supplemented with 5.0 kg/head/day of a 14% protein ration.
composed primarily of processed cereal grains (corn and oats) and cottonseed hulls. Fresh water and minerals were supplied *ad libidum*. Mean body condition score was 6.2 ± 0.5 on a scale from 1 to 10 (1 = emaciated to 10 = obese). This condition score was subjectively given to females to describe overall body condition, fat cover and flesh over the ribs, loin and tailhead (modified from Wagner et al., 1988). Body condition scores based on visual appraisal and palpation of fat covering ranging from 5 to 8 are considered as optimal for normal reproductive function by this laboratory. This study was conducted during the month of April in the years 1999 and 2000.

**Synchronization and Insemination Protocol**

Heifers in this experiment were synchronized and inseminated utilizing the OvSynch protocol first published by Pursley et al. (1995). A timeline of the OvSynch timed-insemination protocol is illustrated in Figure 3.1. Briefly, all females received a 100-µg intramuscular injection of a GnRH analog (Cystorelin®, Merial Limited, Duluth, GA) at the onset of treatment. The purpose of the initial GnRH injection is to induce the ovulation of a dominant follicle and/or induce the emergence of a new follicular wave (Burke et al., 1996). On day 7 of treatment, a 25-mg injection of PGF$_{2\alpha}$ (Lutalyse®, Upjohn Co., Kalamazoo, MI) was administered to regress the corpus luteum, followed 48 hours later by a second intramuscular injection of a GnRH analog (Cystorelin®, Merial Limited, Duluth, GA) to induce ovulation. Timed insemination of all heifers was performed 12 to 16 hours after the second GnRH injection with one straw of frozen-thawed semen from a registered Red Angus bull of known fertility.

An Angus bull was placed with the heifers 10 days post-insemination and was allowed to remain for 45 days. The purpose of the bull was to inseminate any heifers that failed to become pregnant from estrus synchronization and artificial insemination.

**Pregnancy and Sex Determination**

Heifers were palpated *per rectum* between 95 and 100 days post-insemination for pregnancy determination. During the palpation procedure, horn of gestation was recorded for later analysis. Heifers were not included in the study if the uterine horn of gestation could not be determined during palpation. Calving data, including calf sex, were collected on all calves within 24 hours of birth and later compiled with the palpation data collected earlier.
Experiment 3.3

Experimental Animals

Mature primiparous and multiparous, crossbred beef cows were used in this study to evaluate the effect of horn of gestation on the sex ratio of calves. Data for this experiment were collected over a 5-year period across six different crossbred beef herds located throughout the state of Louisiana (Figure 3.2.) These females consisted of various crosses of Angus, Brangus, Brahman, Hereford, Simmental and Gelbvieh breeds, while bulls were primarily of the Angus and Brangus breeds. All cows used in this study were naturally mated during a 60-day breeding season during the months of April and May of each year. During the breeding season, females were maintained in large pastures in groups of 25 to 100 females with one bull for every 25 cows in the breeding herd. Available forage consisted of established bermudagrass (*Cynodon dactylon*) and overseeded ryegrass (*Lolium multiflorum*). Fresh water and minerals were supplied *ad libidum*.

Pregnancy and Sex Determination

Cows were palpated for pregnancy determination approximately 45 days after the end of the breeding season (45 to 110 days of gestation). During the palpation procedure, the horn of gestation was recorded for later analysis. Females in which the horn of gestation could not be determined during palpation were not included in the study. Calving data, including calf sex, were collected on each herd and compiled with the palpation data collected previously.

Statistical Analysis

Data were processed using SAS software Version 9.1.3. In Experiment 3.1, data were analyzed using the Frequency Procedure (Chi Square) (SAS, 2006). The main effects and the differences between uterine horn sides were considered significant at the P<0.05 level. The total number of fetuses and the sex of the fetuses in each uterine horn at the time of examination was used as the end point for side comparison.

Analysis for data from Experiments 3.2 and 3.3, as well as the combined data from all three experiments, was performed using the Glimmix Procedure (SAS, 2006) to analyze logistic regression models with fixed effects. The main effects and the differences between uterine horn sides were considered significant at the P<0.05 level.
Figure 3.1. Timeline of OvSynch protocol for timed insemination of beef heifers (Pursley et al., 1995).
Figure 3.2. Location of research cattle herds in Louisiana used for the collection of sex ratio data in Experiment 3.3.
The total number of calves and the sex of the calves gestated in each uterine horn were used as the end point for side comparison.

**Results**

**Experiment 3.1**

The distribution of male and female fetuses observed in the left and right uterine horns of gravid, abattoir-derived reproductive tracts is summarized in Table 3.1. Of 64 pregnant tracts evaluated, 29 (45.3%) occurred in the left uterine horn while 35 (54.7%) occurred in the right (Figure 3.3). The percentage of pregnancies occurring in the left and right horns was not significantly different.

The overall sex ratio of the fetuses in this study was 53.1% and was not different from the expected sex ratio of 50% (Figure 3.4). However, the sex ratio in the left uterine horn (37.9%) was significantly lower than the sex ratio detected in the right uterine horn (65.7%) (P<0.05). In contrast, the sex ratio detected in the right or left uterine horns was not significantly different from parity.

**Experiment 3.2**

The distribution of male and female calves gestated in the left and right uterine horns of artificially inseminated heifers is summarized in Table 3.2. Of 113 pregnancies evaluated, 53 (46.9%) occurred in the left uterine horn while 60 (53.1%) occurred in the right (Figure 3.5). The percentage of pregnancies occurring in the left and right horns was not significantly different compared with each other and were not different from parity.

The overall sex ratio of the calves born in this study was 50.4% and was not different from parity. (Figure 3.6). The percentage of male and female calves gestated in the left and right uterine horns is illustrated in Figure 3.7. The sex ratio of calves gestated in the left uterine horn (35.8%) was significantly lower than the sex ratio of calves gestated in the right uterine horn (63.3%) (P<0.05). In addition, the sex ratio of calves from the left uterine horn, as well as the calves from the right uterine horn, was significantly different from parity (Figure 3.6).

The sex ratio of calves gestated in the left and right uterine horns during each of the 2 years of this experiment is illustrated in Figure 3.8. The overall sex ratio of calves born in 1999 was 55.6% and did not differ significantly from the overall sex ratio.
Table 3.1. Distribution of male and female fetuses between the left and right uterine horns in gravid, slaughterhouse-derived reproductive tracts.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Uterine Horn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
</tr>
<tr>
<td>No. Pregnant (%)</td>
<td>64 (100)</td>
<td>29 (45.3)</td>
</tr>
<tr>
<td>No. Male (%)</td>
<td>34 (53.1)</td>
<td>11 (37.9)\textsuperscript{a,c}</td>
</tr>
<tr>
<td>No. Female (%)</td>
<td>30 (46.9)</td>
<td>18 (62.1)\textsuperscript{b,c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a-c} Numbers with different superscripts within a column are significantly different (P<0.05).

\textsuperscript{c-d} Numbers with different superscripts within a row are significantly different (P<0.05).

\textsuperscript{*} Numbers with an asterisk differ significantly from parity (P<0.05).
Figure 3.3. Percentage of pregnancies established in the left and right uterine horns of slaughterhouse-derived reproductive tracts. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 3.4. Sex ratio (% male) of fetuses in the left and right uterine horns of slaughterhouse-derived reproductive tracts. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Table 3.2. Distribution of male and female calves gestated in the left and right uterine horns of artificially inseminated heifers.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Uterine Horn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Left</td>
</tr>
<tr>
<td>No. Pregnant (%)</td>
<td>113 (100)</td>
<td>53 (46.9)</td>
</tr>
<tr>
<td>No. Male (%)</td>
<td>57 (50.4)</td>
<td>19 (35.8)\textsuperscript{a,c,*}</td>
</tr>
<tr>
<td>No. Female (%)</td>
<td>56 (49.6)</td>
<td>34 (64.2)\textsuperscript{b,c,*}</td>
</tr>
</tbody>
</table>

\textsuperscript{a-c}Numbers with different superscripts within a column are significantly different (P<0.05).
\textsuperscript{c}Numbers with different superscripts within a row are significantly different (P<0.05).
\textsuperscript{*}Numbers with an asterisk differ significantly from parity (P<0.05).
Figure 3.5. Percentage of pregnancies gestated in the left and right uterine horns of artificially inseminated heifers. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 3.6. Sex ratio (% male) of calves in the left and right uterine horns of artificially inseminated heifers. Bars with different letters differ significantly (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 3.7. Percentage of male and female calves gestated in the left and right uterine horns of artificially inseminated heifers. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 3.8. Sex ratio (% male) of calves gestated in the left and right uterine horns of artificially inseminated heifers by year. a,b Bars with different letters within years are significantly different (P<0.05). c,d Bars with different letters between years are significantly different (P<0.05). *Bars with an asterisk differ significantly from parity (P<0.05).
of calves born in 2000 (47.1%). The sex ratio of right-horn gestated calves was significantly higher than left-horn gestated calves in both 1999 and 2000. However, only the sex ratio of left-horn gestated calves in 2000 was differed significantly from parity.

Experiment 3.3

The distribution of male and female calves gestated in the left and right uterine horns of naturally-mated beef cows is summarized in Table 3.3. Of a total of 2,904 pregnancies evaluated, 1,360 (46.8%) were gestated in the left uterine horn while 1,554 (53.2%) were gestated in the right horn (Figure 3.9). The percentage of pregnancies occurring in the left and right horns were significantly different when compared with each other and differed significantly from the expected equal distribution of 50% (P<0.001).

The overall sex ratio of the calves born in this study was 51.9% and was not different from parity (Figure 3.10). However, the sex ratio of calves gestated in the left uterine horn (34.4%) was significantly lower than the sex ratio of calves gestated in the right uterine horn (67.4%) (P<0.001). In addition, the sex ratio of calves from the left uterine horn and the sex ratio of calves from the right uterine horn were significantly different from parity (P<0.001). The percentage of male and female calves gestated in the left and right uterine horns is illustrated in Figure 3.11.

The sex ratio of calves gestated in the left and right uterine horns during each of the 5 years of this experiment is illustrated in Figure 3.12. The overall sex ratio of calves born in 2002 (54.8%) was significantly greater than parity (P<0.05). In contrast, the overall sex ratio of calves born in years 1998 through 2001 did not differ significantly from parity. The overall sex ratio in 2000 (48.0%) was significantly lower than the overall sex ratios in 2001 and 2002 (53.3% and 54.8%, respectively) but did not differ compared with 1998 (49.8%) and 1999 (52.5%). However, the overall sex ratios of calves born in 2001 and 2002 were not significantly different from the overall sex ratio of calves born in both 1998 and 1999.

The sex ratio of right-horn gestated calves was significantly higher than the sex ratio of left-horn gestated calves during all 5 years of the experiment. In addition, the sex ratios of both the left- and right-horn gestated calves were significantly different from parity in all five years.
Table 3.3. Distribution of male and female calves gestated in the left and right uterine horns of naturally mated beef cows.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Uterine Horn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
</tr>
<tr>
<td>No. Pregnant (%)</td>
<td>2,904 (100)</td>
<td>1,360 (46.8)(^{c,\ast})</td>
</tr>
<tr>
<td>No. Male (%)</td>
<td>1,508 (51.9)(^a)</td>
<td>468 (34.4)(^{a,c,\ast})</td>
</tr>
<tr>
<td>No. Female (%)</td>
<td>1,396 (48.1)(^b)</td>
<td>892 (65.6)(^{b,c,\ast})</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Numbers with different superscripts within a column are significantly different (P<0.001).

\(^{c,d}\)Numbers with different superscripts within a row are significantly different (P<0.001).

\(^\ast\)Numbers with an asterisk differ significantly from parity (P<0.001).
Figure 3.9. Percentage of pregnancies gestated in the left and right uterine horns of naturally mated, crossbred beef cows.  

Bars with different letters are significantly different (P<0.001).  Bars with an asterisk differ significantly from parity (P<0.001).
Figure 3.10. Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows. \(^{a,b}\)Bars with different letters are significantly different (P<0.001). \(^{\ast}\)Bars with an asterisk differ significantly from parity (P<0.001).
Figure 3.11. Percentage of male and female calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows. Bars with different letters within sides are significantly different (P<0.001). Bars with an asterisk differ significantly from parity (P<0.001).
Figure 3.12. Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows by year. a,b Bars with different letters within years are significantly different (P<0.05). c,d Bars with different letters between years are significantly different (P<0.05). * Bars with an asterisk differ significantly from parity (P<0.05).
The sex ratio of calves gestated in the left and right uterine horns at each of the six locations is illustrated in Figure 3.13. The overall sex ratio of calves born at Locations 3 and 4 differed significantly from parity (P<0.05). In contrast, the overall sex ratio at the remaining locations failed to differ from parity. The overall sex at Location 6 (41.7%) was significantly lower than the overall sex ratios at Locations 2, 3 and 4 (54.5%, 52.4% and 52.0%, respectively) but did not differ compared with Location 1 (48.1%) and Location 5 (50.0%). However, the overall sex ratios of calves born at Locations 1 and 5 were not significantly different from the overall sex ratio of calves born at Locations 2, 3 and 4.

The sex ratio of calves gestated in the left uterine horn at Location 5 was significantly lower compared with the sex ratio of left-horn gestated calves at Locations 1, 2, 3, 4 and 6. In addition, the sex ratios of both the left- and right-horn gestated calves were significantly different from parity at Locations 1, 2, 3 and 4. In contrast, the sex ratios of left-horn gestated and right-horn gestated calves failed to differ significantly from parity at Locations 5 and 6.

**Combined Experiments**

The combined distribution of male and female fetuses observed in the left and right uterine horns from Experiment 3.1, 3.2 and 3.3 is summarized in Table 3.4. A total of 3,081 pregnancies were evaluated in the three studies. Of the 3,081 pregnancies, 1,442 (46.8%) were gestated in the left uterine horn compared with 1,639 (53.4%) right horn pregnancies (Figure 3.14). The percentage of pregnancies occurring in both the left and right uterine horns was significantly different from parity. The overall sex ratio of the fetuses and calves in the three experiments was 51.9% and was not different from parity (Figure 3.15). However, the sex ratio in the right uterine horn (67.2%) was significantly higher than the sex ratio recorded in the left uterine horn (34.5) (P<0.001). In addition, the sex ratio detected in the right and left uterine horns were significantly different from parity.

**Discussion**

The number of studies conducted to evaluate the asymmetric distribution of male and female fetuses between the left and right uterine horns is small and is limited to polytocous laboratory species. Based on the current literature, this is the first
Figure 3.13. Sex ratio (% male) of calves gestated in the left and right uterine horns of naturally mated, crossbred beef cows by location. 

Bars with different letters within locations are significantly different (P<0.05). Bars with different letters between locations are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Table 3.4. Combined distribution of male and female pregnancies gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Uterine Horn</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
<td></td>
</tr>
<tr>
<td>No. Pregnant (%)</td>
<td>3,081 (100)</td>
<td>1,442 (46.8)</td>
<td>1,639 (53.2)</td>
<td></td>
</tr>
<tr>
<td>No. Male (%)</td>
<td>1,599 (51.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>498 (34.5)&lt;sup&gt;a,c,*&lt;/sup&gt;</td>
<td>1,101 (67.2)&lt;sup&gt;a,d,*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>No. Female (%)</td>
<td>1,482 (48.1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>944 (65.5)&lt;sup&gt;b,c,*&lt;/sup&gt;</td>
<td>538 (32.8)&lt;sup&gt;b,d,*&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers with different superscripts within a column are significantly different (P<0.001).

<sup>c-d</sup>Numbers with different superscripts within a row are significantly different (P<0.001).

<sup>∗</sup>Numbers with an asterisk differ significantly from parity (P<0.001).
Figure 3.14. Percentage of pregnancies gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3. Bars with different letters are significantly different (P<0.001). Bars with an asterisk differ significantly from parity (P<0.001).
Figure 3.15. Sex ratio (% male) of fetuses and calves gestated in the left and right uterine horns in Experiments 3.1, 3.2 and 3.3. Bars with different letters are significantly different (P<0.001). Bars with an asterisk differ significantly from parity (P<0.001).
investigation to evaluate the sex ratio of calves gestated in the left and right uterine horns in cattle.

Numerous studies have demonstrated lateral asymmetries in the cow. The CL is more often found in the right ovary than in the left in cycling heifers (Reese and Turner, 1938). Similarly, Rajakoski (1959) reported a significantly higher number of follicles ≥5 mm in the right ovary than in the left in mature cows. These asymmetries in cattle were corroborated by Perkins et al. (1954) and Scanlon (1972) with reports of more ovulations from the right ovary compared with the left ovary. In addition, Reese and Turner (1938) reported that a significantly greater proportion of pregnancies were gestated in the right uterine horn (66.1%) compared with the proportion of left-horn pregnancies.

The present results demonstrated that, in heifers and cows, the sex ratios of calves gestated in the left and right uterine horns are significantly different (P<0.001) and are also different from parity (P<0.001). The sex ratio of calves and fetuses gestated in the right horn was significantly higher than the sex ratio of left-horn gestated calves and fetuses. These results compare favorably with those previously reported for the mouse (Endo et al., 1987) and Mongolian gerbil (Clark and Galef, 1991), but were the reverse of the fetal sex ratio distribution observed in the uterus of the rabbit (YoungLai, 1981).

In the mouse and rabbit, the underlying mechanisms that might be responsible for the altered distributions were not investigated. However, in the Mongolian gerbil, Clark et al. (1994) demonstrated that the ovary of origin influenced the sex of the fetus rather than the horn of gestation. This conclusion was reached after the sex ratio of fetuses observed in the left and right uterine horns of normal females was reversed in females in which the left and right ovaries were surgically translocated. The authors postulated that “ovarian factors” were responsible for the distorted sex ratios detected. Transuterine migration, in which embryos disseminate throughout the uterus, has not been previously described in the Mongolian gerbil. However, given the altered sex ratios detected in the left and right uterine horns in normal females, and in ovarian translocated individuals, the probability of transuterine migration occurring in this species appears to be relatively low.
The results from the present experiments indicate that the ovary of origin may also influence the sex of the offspring in cows. However, even though transuterine migration has been reported as an extremely infrequent occurrence in cattle (0 to 3%) (Boyd et al., 1944; Perkins et al., 1954; Scanlon, 1972), the same conclusion cannot be reached in cattle based solely on the results of the present experiments. Because the number of matings prior to the pregnancies investigated in this experiment, and the sex of any possible pregnancies remains unknown, sex-specific embryonic mortality within individual uterine horns to achieve a pregnancy of the preferential sex remains a possibility.

A similar phenomenon has been described in the nutria (Myocastor coypu), in which female-biased litters were aborted by young females and larger, male-biased litters usually resulted after the female was mated again (Gosling, 1998). However, this obstacle can easily be circumvented in cattle by determining the uterine horn of gestation in the gravid female after a known mating and determining the gestational length, as well as the sex of the offspring.

Trivers and Willard (1973) originally suggested that alteration of the sex ratio might be accomplished through parental manipulation of postnatal mortality, suggesting that mothers should prematurely terminate investment in offspring that were less likely to breed successfully or fail to achieve their maximum reproductive potential. Likewise, manipulation of the sex ratio before birth, as hypothesized by Maynard-Smith (1980), would be preferential and would minimize reproductive inefficiencies.

The results from the present experiments demonstrated that a significantly greater proportion of male calves are gestated in the right uterine horn and a greater proportion of female calves in the left horn of domestic cattle. However, it remains unclear if the ovary of origin influences the sex of the calf, or if selection pressures applied by the uterus resulting in sex-specific embryonic mortality alters the observed sex ratio. Further investigations into the underlying mechanisms responsible for the skewed sex ratios in the left and right uterine horns found in the present experiments are warranted. Assisted reproductive technologies such as IVF and ET, as well as advanced biotechnologies like PCR, can provide insight into these mechanisms in a
more time-efficient manner as compared with the methodologies employed in the present experiments.
CHAPTER IV

SEX RATIO OF EMBRYO TRANSFER CALVES PRODUCED FROM EMBRYOS RECOVERED FROM SUPEROVULATED DONORS AND TRANSFERRED TO THE LEFT AND RIGHT UTERINE HORMS OF RECIPIENTS

Introduction

Research in the rabbit (YoungLai, 1981) and Mongolian gerbil (Clark and Galef, 1990) has indicated a skewed sex ratio within the left and right uterine horns of the gravid female. Clark et al. (1994) postulated that the ovary of origin was responsible for the skewed ratio, given that the ratio followed the ovary when translocated within the same animal. The results of the previous experiment indicate an altered sex ratio of calves and fetuses gestated within the left and right uterine horns of cattle. However, it remains unclear if the altered sex ratios are the result of increased mortality of one sex within the uterus or, as hypothesized by Clark et al. (1994), the ovary of origin of the oocyte provides preferential selection for either X- or Y-chromosome bearing sperm at fertilization.

In numerous species, preferential survival of one sex within the uterus has been described (Hafez, 1965). For example, in pigs, the sex ratio has been reported to increase as litter size increases (Perry, 1960; Anderson, 1978). The disparity observed in the sex ratio was attributed to increased mortality of females caused by reduced uterine space as a result of the increased development of male fetuses. However, no alteration of the sex ratio was reported in the mouse (Perry, 1971) or guinea pig (Peaker and Taylor, 1996) as litter size increased.

Embryo transfer provides a relatively straightforward method to illuminate uterine horn selection pressures applied to male or female embryos. While the overall sex ratio of calves produced from embryo transfer has been reported to be slightly higher than parity (Elsden et al., 1979; Anderson, 1983; King et al., 1985), the sex ratio of calves resulting from transfers to specific uterine horns remains unknown. An altered sex ratio from the left and right uterine horns may indicate uterine selection pressures providing a preferential advantage to embryos of one sex.

Therefore, the objective of the present experiment was to analyze embryo transfer records and the resulting calving data to determine the percentage of male and
female calves produced from transfers to the left and right uterine horns of recipient cattle to determine if the horn of transfer influences the sex of the calf.

**Materials and Methods**

**Experimental Animals**

A retrospective analysis was performed utilizing embryo transfer and calving records from two cattle ranches in Brazil. Nelore donors maintained at Fazenda Nelore, owned by Grupo Marata and located in Santa Ines, and Fazenda Lago Azul, owned by GM Agropecuaria, located in Igarape do Meio, were used for the production of embryos. The ranches are located in the state of Maranhão in northeast Brazil and are separated by ~30 km (Figure 4.1). The climate in this region is classified as semi-humid tropical with a mean annual temperature of 27.2°C and an average annual rainfall of 159.8 cm. Recipients, managed at the same locations along with the donors, consisted of *Bos indicus*-cross females, primarily F1 crosses of Holstein and Gyr. Donors and recipients were maintained on forage pastures, mostly *Brachiara brizantha*, with donors receiving supplementation with concentrated feeds prior to stimulation. Climate and feed conditions for both donors and recipients were similar at both locations.

**Superovulation Protocol**

A timeline of the superovulation protocol used for the production of embryos is illustrated in Figure 4.2. At the onset of treatment, all females received a 15-mg injection of PGF2α (Preloban®, Intervet International, Germany) to induce estrus. A 3-mg norgestomet ear implant (Crestar®, Intervet International, Holland) was inserted 7 days (Treatment day 8) following the initial PGF2α injection. All donors received a 2.5-mg intramuscular injection of estradiol benzoate (EB) (Estrogin®, Farmavet Produtos Veterinarios, Brazil) on Treatment day 9. A total of 237.5 IU of porcine FSH (Pluset®, Laboratorios Callier, Spain) was delivered in seven intramuscular injections over 4 days in descending doses, administered ~12 hours apart. A 15-mg injection of PGF2α (Preloban®, Intervet International, Germany) was administered with the fifth and sixth FSH injections and the norgestomet ear implant was removed at the time of the sixth FSH injection.

Donor females were observed for estrus behavior every 2 hours beginning approximately 6 hours after the final FSH injection. Estrus was expected on the morning
Figure 4.1. Location of farms used for the collection of embryo transfer and calving data in the state of Maranhão in northeast Brazil.
Figure 4.2. Timeline of the superovulation protocol for Nelore donor cattle.
after the seventh FSH injection. Females that failed to exhibit estrus at the expected
time were administered an 84-µg intramuscular injection of buserelin acetate
(Conceptal®, Intervet International, Germany) 48 hours after the primary PGF₂α (morning
of Treatment day 15). Donors exhibiting estrus behavior were inseminated artificially 12
hours after the onset of estrus with two straws of semen and again at 24 hours post-
estrus with one straw of semen. Donors that failed to exhibit estrus were artificially
inseminated 12 hours after the buserelin injection with two straws of semen and again at
24 hours with one straw of semen. Embryos were recovered nonsurgically from all
donors 7 days after the onset of estrus or buserelin acetate injection.

**Embryo Collection Procedure**

Using a procedure similar to the techniques reported by Drost et al. (1976),
Elsden et al. (1976), Rowe et al. (1976) and Greve et al. (1977), a nonsurgical embryo
recovery was performed on each donor on day 7 following the onset of standing estrus,
as determined by visual observation. Prior to embryo collection, females were
restrained in a standard cattle squeeze chute and administered a 5-ml epidural injection
of lidocaine (Lidocaine Hydrochloride 2%). The ovaries of all animals were palpated per
rectum to estimate the number of corpora lutea and follicles present on each ovary.

At the time of collection, a two-way Foley catheter (16 to 18 Fr.) with stiffening
stylette was guided through the cervix, past the internal os of the cervix into the body of
the uterus, where the cuff was inflated with 5 to 10 ml of flush medium. To prevent
back-flow of medium, the cuff of the catheter was seated securely against the internal
cervical os with slight rearward pressure. A commercial embryo collection medium
(DMPBS Flush®, Nutricell, Brazil) supplemented with 0.3% FCS was used as the
flushing medium for the recovery of embryos. The uterine horns were simultaneously
and repeatedly flushed with 1 to 2 liters of medium. Embryo collection medium
recovered from the uterus was filtered using a 75-µm, inline filter (Milipore®, São Paulo,
Brazil). At the completion of the flushing procedure, the cuff was deflated and the
catheter was slowly removed and drained into the filter.

The filtered flushing medium was then placed into 100 mm x 15 mm petri dishes
(Falcon®, Beckton-Dickinson & Co., Franklin Lakes, NJ) maintained at room
temperature. Once visualized under microscopy, embryos were transferred into a
commercial embryo holding medium (TQC Holding Plus®, Nutricell, Brazil) by means of a capillary pipette (Unopette®, Becton Dickinson & Co., Rutherford, NJ) and held for a period of 5 to 7 hours at room temperature until transfer into recipients. Prior to transfer, individual embryos in holding medium were loaded into 0.25-ml straws.

**Embryo Transfer**

Embryos were transferred to recipients nonsurgically using a method similar to those reported by Trounson et al. (1978) and Rowe et al. (1980). Briefly, recipient females were restrained in a standard squeeze chute and palpated for ovarian activity and the presence of a corpus luteum (CL). Recipients determined to have an acceptable CL present on an ovary were administered a 5-ml injection of lidocaine (Lidocaine Hydrochloride 2%) into the first intercoccygeal space as a caudal epidural block. Straws containing the embryos were loaded into a Cassou transfer gun (IMV, France) and a steel-tipped, side delivery sheath (IMV, France) was placed over the gun. A plastic chemise was placed over the transfer gun to minimize contamination risk.

The transfer gun with the chemise was inserted in the vagina and slowly guided to the cervix. Once at the external os of the cervix, the chemise was pulled to allow passage of the transfer gun tip. The transfer gun, without the chemise, was then manipulated through the cervix and gently guided past the greater curvature of the uterine horn ipsilateral to the ovary with the CL. Each embryo was quickly deposited in the distal portion of the uterine horn. The uterine horn to which the embryo was transferred was recorded for later analysis. Calving data, including calf sex, was collected within 24 hours of birth and compiled with the embryo transfer data previously recorded.

**Statistical Analysis**

Data were processed using SAS software Version 9.1.3. Analysis of the data from this experiment were performed using the Glimmix Procedure (SAS, 2006) to analyze logistic regression models with fixed effects. The main effects and the differences between uterine horn sides were considered significant at the P<0.05 level. The total number of calves and the sex of the calves produced from transfers to each uterine horn side were used as the end point for uterine horn side comparison.
Results

The distribution of male and female calves resulting from the transfer of embryos to the left and right uterine horns of recipients is summarized in Table 4.1. Of a total of 521 pregnancies evaluated, 202 (38.8%) resulted from left horn transfers, and was significantly lower (P<0.001) than the 319 (61.2%) calves produced from right horn transfers (Figure 4.3).

The overall sex ratio of calves produced was 50.3% and was not significantly different from parity. The sex ratio of calves derived from left horn transfers (50.0%) was not significantly different from the sex ratio of calves from right horn transfers (50.5%) (Figure 4.4). No significant difference was detected between the sex ratio of left horn transfers or the sex ratio of right horn transfers compared with parity.

The overall sex ratios of calves produced at both locations were not significantly different (48.5% vs. 52.1%) and did not differ from parity (Figure 4.5). The sex ratio of calves produced from right horn transfers compared with left horn transfers did not differ at either location.

Discussion

The results from this experiment compare favorably with those previously reported in the literature. Significantly more calves were produced from right horn transfers than from left horn transfers. Previous studies have indicated that cattle ovulate from the right ovary more than from the left ovary (Reece and Turner, 1938; Boyd et al., 1944; Perkins et al., 1954; Scanlon, 1972; DelCampo et al., 1977). Given that embryos were transferred to the uterine horn ipsilateral to the ovary containing the CL in recipients, a greater number of right-horn derived calves is not surprising, though the percentage documented in this experiment (61.2%) is greater than the generally accepted consensus of 54% to 55%. However, in a study of slaughterhouse reproductive tracts, Reece and Turner (1938) found that 66.1% of pregnancies in cows were gestated in the right uterine horn.

The overall sex ratio of 50.3% of ET calves produced in the present study compares favorably with the sex ratio of naturally sired calves previously reported in the scientific literature, as well as with the sex ratios observed in Experiment 3.3. However, the sex ratio of calves produced from embryo transfer has been reported to be
Table 4.1. Distribution of male and female calves resulting from the transfer of embryos to the left and right uterine horns of recipients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Left (%)</th>
<th>Right (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Pregnant (%)</td>
<td>521 (100)</td>
<td>202 (38.8)^c,∗</td>
<td>319 (61.2)^d,∗</td>
</tr>
<tr>
<td>No. Male (%)</td>
<td>262 (50.3)</td>
<td>101 (50.0)</td>
<td>161 (50.5)</td>
</tr>
<tr>
<td>No. Female (%)</td>
<td>259 (49.7)</td>
<td>101 (50.0)</td>
<td>158 (49.5)</td>
</tr>
</tbody>
</table>

^a^bNumbers with different superscripts within a column differ significantly (P<0.05).

^c^dNumbers with different superscripts within a row differ significantly (P<0.001).

∗Numbers with an asterisk differ significantly from parity (P<0.001).
Figure 4.3. Percentage of calves resulting from embryos derived from superovulated donors and transferred to the left and right uterine horns of recipients. Bars with different letters are significantly different (P<0.01). Bars with an asterisk differ significantly from parity (P<0.05).
<table>
<thead>
<tr>
<th>Horn of Transfer</th>
<th>Overall</th>
<th>Left</th>
<th>Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex Ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.4. Sex ratio (% male) of calves resulting from the transfer of embryos to the left and right uterine horns of recipients. *Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 4.5. Sex ratio (% male) of calves resulting from the transfer of embryos to the left and right uterine horns of recipients by location. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
significantly greater than parity (Elsden et al., 1979; Anderson, 1983; King et al., 1985) and greater than the 50.3% found in this study. The overall sex ratios at both farms were not different and, considering that donors were maintained in similar environments at both locations, no significant difference was expected.

The sex ratios of ET calves produced from both left- and right-horn transfers (50.0% and 50.5%, respectively) were not significantly different and did not differ from parity. An altered sex ratio of calves from the left and right uterine horns, similar to the results detected in Experiments 3.1, 3.2 and 3.3, would be expected if the uterine horns provided a preferential environment to either male or female embryos. A low incidence of transuterine migration of transferred embryos in recipient cattle has been reported (McMillan and Peterson, 1999), minimizing the likelihood of embryos of each sex migrating to a preferred uterine horn for gestation.

Clark et al. (1994) postulated that in the Mongolian gerbil, the ovary of origin of the oocyte influenced the sex of the resulting fetus as opposed to the uterine horn of gestation. These data, in combination with the results from Experiments 3.1, 3.2 and 3.3 support this hypothesis in cattle.

Data from the present study indicate than there is no preferential selection for embryos of a single sex in the uterine horns of recipient cattle. These results, in addition to the results from the previous experiment, indicate that the ovary of origin from which the pregnancy is derived, rather than the uterine horn of gestation, may influence the sex of offspring in cattle.
CHAPTER V

SEX RATIO OF EMBRYOS RECOVERED FROM THE LEFT AND RIGHT UTERINE HORNS OF SUPEROVULATED BEEF COWS

Introduction

While Clark and Galef (1990) reported a greater proportion of males gestated in the right uterine horn of Mongolian gerbils and a greater proportion of females in the left, Clark et al. (1994) determined that the the ovary of origin, rather than the horn of gestation, influenced the sex of the fetus. The authors arrived at this conclusion after the sex ratio of the fetuses in the left and right uterine horns followed the ovaries in females in which the ovaries were surgically translocated. While transuterine migration has not been previously described in the Mongolian gerbil, reports that the uterine horn sex ratio differs from parity in both normal and translocated-ovary individuals suggest that little or no transuterine migration exists in this species.

Transuterine migration occurs with high frequency in litter producing species such as the pig (Dhindsa et al, 1967; Polge and Dziuk, 1970; Pope et al., 1986), as well as in species that regularly produce twins and higher order births, such as sheep (Boyd, 1944; Abnes and Woody, 1971; Scanlon, 1972; Doney et al., 1973) and goats (Lyngset 1968, Mani et al., 1992). In cattle, a migration frequency of 1 to 3% has been reported (Boyd et al., 1944; Perkins et al., 1954; Olds and Vandemark, 1957), while no migration was reported in a large study by Scanlon and Peterson (1970). In addition, a relatively low transuterine migration frequency has also been reported in superovulated cattle (Hafez,1964). Given the low incidence of transuterine migration reported in cattle, embryos recovered from the uterine horns can provide indirect evidence about the ovary ipsilateral to the uterine horn from which the embryos are recovered.

The sex ratio of calves produced from ET has been reported to be greater than parity (Elsden et al., 1979; King et al., 1985). Because the sex ratio of ET calves is measured at birth, it remains unclear if the elevated sex ratio is the result of increased male embryo production in donors or is the result of increased pregnancy potential of male embryos. In addition, the sex ratio of embryos recovered from individual uterine horns remains unknown.
Therefore, the objective of this study is to determine the sex ratio of embryos recovered from the left and right uterine horns of superovulated beef cows. Considering that the embryos are produced from a known mating and the low incidence of transuterine migration in cattle, the sex ratio of the embryos should provide accurate insight into the contribution of the ovary of origin in influencing the sex of the resulting embryos.

**Materials and Methods**

**Experimental Animals**

A group of 20 Brahman, Continental and European breed crossbred females with estrous cycles of normal-length were used in the present study to evaluate the sex ratio of embryos recovered from the left and right uterine horns of superovulated donors. Primiparous and multiparous animals were randomly selected from a large herd maintained at the Louisiana Agricultural Experiment Station located in St. Gabriel, Louisiana (see Figure 3.2). The study was conducted during the period of February to April, 2004. During this period, two embryo recovery replicates were performed with 10 cows stimulated in each replicate.

Experimental animals were maintained in large pastures with bermudagrass hay, minerals and fresh water supplied *ad libidum*. All females were in excellent health and good body condition at the onset of treatment. Animal age ranged from 3 to 12 years with a mean ($\pm$SD) of 7.8$\pm$2.2. Body weights ranged from 410 to 656 kg with a mean of 502.3$\pm$71.4 kg. Mean body condition score was 6.2$\pm$0.4 on a scale from 1 to 10 (1 = emaciated to 10 = obese). This condition score was subjectively assigned to cows to describe overall body condition, including fat cover and flesh over the ribs, loin and tailhead (modified from Wagner et al., 1988). Body condition scores based on visual appraisal and palpation of fat covering ranging from 6 to 8 are considered as optimal for normal reproductive function by this laboratory.

**Superovulation Protocol**

A timeline of the superovulatory protocol used for the production of embryos in this experiment is illustrated in Figure 5.1. Prior to superovulatory treatment, all females were palpated *per rectum* and ultrasonically scanned for normal ovarian activity. At the onset of treatment, all females received a 25-mg injection of PGF$_{2\alpha}$ (Lutalyse®, Upjohn
Figure 5.1. Timeline of the superovulation protocol for crossbred beef donor cattle.
Donors that exhibited estrus began superstimulation treatment 7 to 9 days following estrus.

A dominant follicle removal (DFR) via ultrasound guided follicular aspiration of all follicles >5 mm was performed ~48 hours prior to the beginning of superstimulation treatment in all donors. A total of 32 mg of porcine FSH (Sioux Biochemical, Sioux Center, IA) was administered in eight injections over 4 days in descending-dose, twice-daily intramuscular injections. A 25-mg injection of PGF\textsubscript{2a} (Lutalyse\textsuperscript{®}, Upjohn Co., Kalamazoo, MI) was administered with the sixth and seventh FSH injections. Experimental animals were observed twice daily, at dawn and dusk, for estrus behavior. Estrual activity of all animals was also monitored with HeatWatch\textsuperscript{®} system (DDx, Inc., Denver, CO). For this experiment, estrus parameters were defined as three mounts within a 2-hour period, with a minimum duration of 3 seconds per mount. Estrus was expected on the morning after the eighth FSH injection. Donors were artificially inseminated at 12 hours and again at 24 hours after the onset of estrus, with one straw of semen from a Holstein bull of known fertility at each insemination. Embryos were nonsurgically recovered from donors 7 days after the onset of estrus (estrus = day 0).

**Embryo Collection Procedure**

On day 7 following the onset of standing estrus, as determined by the HeatWatch\textsuperscript{®} system (DDx, Inc., Denver, CO), a nonsurgical embryo recovery was performed on each donor, following a similar procedure to those reported by Drost et al. (1976), Elsdon et al. (1976), Rowe et al. (1976) and Greve et al. (1977). Prior to embryo collection, females were restrained in a standard cattle squeeze chute and administered 15 mg of acepromazine (Prom Ace\textsuperscript{®}, Fort Dodge Laboratories, Fort Dodge, IA) and a 5-ml epidural injection of lidocaine (Lidocaine Hydrochloride 2%, Butler Columbus, OH). Prior to collection, the ovaries of all animals were palpated per rectum and ultrasonically checked to determine the number of CL and follicles present on each ovary.

For embryo collection, a two-way Foley catheter (16 to 18 Fr.) with stiffening stylette was guided through the cervix into the right uterine horn. Once the tip of the catheter was positioned at the greater curvature of the uterine horn, the cuff was inflated with 7 to 10 ml of flush medium to stabilize the uterine position and prevent back-flow. Embryo collection medium (Appendix A) was used to flush the embryos from the uterine
horns in this experiment. Each uterine horn was repeatedly flushed with 500 to 750 ml of medium. When flushing of the right horn was completed, the cuff was deflated and the catheter was then removed and placed in the left uterine horn, which was then similarly flushed with approximately the same volume of flushing medium.

The filtered flushing medium was then placed into 100-mm x 15-mm petri dishes (Falcon®, Beckton Dickinson & Co., Franklin Lakes, NJ) and maintained at room temperature (25°C). A separate observer performed all embryo searches using a stereomicroscope. Once detected, embryos were transferred to embryo holding medium (Appendix B) by means of a capillary pipette (Unopette®, Becton Dickinson & Co., Rutherford, NJ).

Embryos from each donor were evaluated at magnification (100X) under phase-contrast microscopy. Embryo quality (EQ) grades, ranging from 1 to 4 (1 = excellent to 4 = poorest quality), were subjectively assigned to each embryo, as previously characterized by Elsden et al. (1978) and Wright (1981). In this study, subjective grading of bovine embryos was based on symmetrical appearance of the embryo, presence of extruded blastomeres, degeneration of the cell mass and developmental synchrony to day of cycle of the donor as described by Elsden et al. (1978) and Shea (1981).

Ova were considered fertilized if the presences of cleavage divisions were apparent. Furthermore, embryos were classified as transferable quality if they were of an excellent, good or fair morphological condition (EQ = 1, 2 or 3, respectively), as described by Wright (1981). Based on the developmental stage and quality grade, embryos were assigned a modified RED score (mRED). The mRED score was adapted from the Ryan Embryo Development score originally reported by Ryan et al. (1992) for use with bovine embryos cultured in vitro (Table 5.1).

Prior to the onset of this study, two criteria were established for the inclusion of a donor female in the results of this experiment. First, each potential donor must have exhibited standing estrus. Secondly, each donor must have responded to FSH treatment by producing two or more CL. Any donor not meeting these criteria was not included in the results. After grading was completed, embryos were cryopreserved for sexing at a later date.
Table 5.1. The modified Ryan Embryo Development (mRED) rating system.\(^1\)

<table>
<thead>
<tr>
<th>Embryo quality(^2)</th>
<th>Compacted morula</th>
<th>Early blastocyst</th>
<th>Blastocyst</th>
<th>Expanded blastocyst</th>
<th>Hatched blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>2.00</td>
<td>3.00</td>
<td>4.00</td>
<td>5.00</td>
</tr>
<tr>
<td>2</td>
<td>0.66</td>
<td>1.66</td>
<td>2.66</td>
<td>3.66</td>
<td>4.66</td>
</tr>
<tr>
<td>3</td>
<td>0.33</td>
<td>1.33</td>
<td>2.33</td>
<td>3.33</td>
<td>4.33</td>
</tr>
<tr>
<td>4</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^1\)Adapted from Ryan et al. (1992).

\(^2\)Embryo quality grades (1 = excellent to 4 = degenerate).
Embryos to be frozen were placed in 1.4 M glycerol freezing medium (Appendix C), loaded into labeled 0.25-ml straws and held at room temperature for 10 minutes. The straws containing individual embryos were placed into a Planer R204 cell freezer (Planer Products, Ltd., Surrey, UK) maintained at -7°C with liquid nitrogen (LN2) vapor. Embryos were held at -7°C for 2 minutes, seeded by touching supercooled forceps to the uppermost column of freezing medium and then held for an additional 8 minutes at -7°C. The embryos were subsequently cooled to -36°C at -0.5°C per minute. Upon completion of the freezing program, the straws were plunged into LN2. Groups of straws containing embryos were placed in labeled goblets, which were then placed on aluminum canes and placed in dewar tanks containing LN2 for storage until the embryo sexing procedure was performed.

To thaw, straws containing frozen embryos were exposed to air for 10 seconds and held in a 30°C water bath for an additional 10 seconds. Upon expulsion from the straw, embryos were placed in 1M sucrose thaw medium (Appendix D) for 15 minutes at room temperature to allow rehydration. After rehydration, embryos were placed in holding medium (Appendix B) to await sexing.

**Embryo Sex Determination**

Embryos were sexed using a modified protocol based on the bovine embryo sexing kit supplied by AB Technology (AB Technology, Inc., Pullman, WA). The kit, through the use of polymerase chain reaction (PCR), is designed to detect male specific, Y-chromosomal DNA sequences (Mathews et al., 1987; Bondioli et al., 1989) and is based on the micromanipulation methodologies of Herr and Reed (1991). In the present experiment, the entire embryo was used in the PCR reaction rather than a biopsy of 8 to 10 cells removed from the trophectoderm.

Following rehydration, individual embryos were washed three times through phosphate-buffered saline containing 2 mg/ml polyvinylpyrrolidone (PBS-PVP) to remove serum. In an effort to reduce the probability of DNA contamination from ancillary sperm, the zona pellucida of each embryo was removed by repeated, rapid pipetting of the embryo through a small bore (~50 µm), fire-polished glass pipette after the final wash. Following zona removal, the embryo in 2 µl of PBS-PVP was placed in a 200-µl Eppendorf microcentrifuge tube containing 8 µl of ddH2O. After addition of the embryo,
the assay tubes were immediately plunged in LN2, then held in an ice bath while additional embryos were processed. Once a pool of 15 to 20 embryos had been processed, 12 µl of YCD® (Y-Chromosome Determinant) containing Taq polymerase, dNTP’s, male-specific (Y chromosome) and nonmale specific (autosomal) probes, was added to the assay tube and thoroughly mixed. The reactions were thermocycled in a PTC-150 minicyler (MJ Research, Waltham, MA). The thermocycler profile consisted of 1 minute at 95°C, followed by 33 cycles of 10 seconds at 95°C, 20 seconds at 64°C and 15 seconds at 72°C and held at 4°C following completion of the last cycle.

Following thermocycling, 5 µl of gel loading buffer (Appendix E) was mixed with the contents of each assay tube, and 18 to 20 µl of the PCR product was loaded into the designated wells of a 3% agarose gel (Appendix F) in a BioMax MP1015 electrophoresis unit (Kodak Imaging Systems, New Haven, CT). Electrophoresis of the gels was run at 140 volts for 20 minutes. DNA bands were visualized by ethidium bromide fluorescence using ultraviolet illumination.

For each gel electrophoresis, a negative control to detect contamination without embryo or genomic DNA was processed. Male and female controls, consisting of peripheral blood lymphocytes, were also included in each gel. The presence of a nonspecific DNA band indicated that a viable sample was present in the assay tube, while primer bands were occasionally visualized in lanes depending on the number of cells present in that particular embryo. The presence of a male-specific band identified male samples, while female samples were identified by the absence of this band under UV illumination (Figure 5.2).

**Statistical Analysis**

Data in this experiment were processed using SAS software Version 9.1.3 and analysis was performed using the Glimmix Procedure (SAS, 2006) to evaluate logistic regression models with fixed effects. The main effects and the differences between uterine horn sides were considered significant at the P<0.05 level. The total number of embryos and the sex of the embryos recovered from each uterine horn, as well as the quality and developmental stage of the embryos, were used as the end points for side and sex comparisons. The values for mRED Score are expressed as mean (±SEM) per classification. A P<0.05 value was considered significant.
Figure 5.2. Agarose gel under UV illumination after electrophoresis for sex determination of bovine embryos.
Results

The distribution of male and female embryos collected from the left and right uterine horns of superovulated beef cows is summarized in Table 5.2. A total of 205 embryos were collected from 20 donor cows, with a mean of 10.25 embryos collected from each donor. Of the 205 embryos, 111 (54.1%) were recovered from the right uterine horn while 94 (45.9%) were recovered from the left uterine horn (Figure 5.3). The number of embryos recovered from the left and right horns was not significantly different.

The overall sex ratio of the embryos recovered in this study was 52.7% and was not different from the expected sex ratio of 50% (Figure 5.4). However, the sex ratio of the embryos from the left uterine horn (45.7%) was significantly lower than the sex ratio of embryos from the right uterine horn (58.6%) (P=0.045). In contrast, the sex ratio of the embryos recovered from the right and left uterine horns was not significantly different from parity. The distribution of male and female embryos recovered from the left and right uterine horns is shown in Figure 5.5.

The sex ratio of embryos collected from the left and right uterine horns during each replicate is illustrated in Figure 5.6. The overall sex ratio of the embryos recovered in Replicate 1 and Replicate 2 was not significantly different. In addition, the sex ratio of embryos from the right and left horns in Replicate 1 were not significantly different. However, in Replicate 2, the sex ratio of embryos from the right horn was significantly higher compared with the sex ratio of the left horn. The overall sex ratio, as well as the sex ratio of embryos from both the left and right uterine horns did not differ from parity in either replicate.

The mean mRED Score values for male and female embryos collected from the left and right uterine horns is summarized in Table 5.3. Overall, the mean mRED Score for male and females embryos from the left and right horns in this experiment was 0.90±0.03. In addition, the overall mRED Scores for male embryos and female embryos collected from both uterine horns was 0.91±0.05 and 0.90±0.05, respectively, and was not significantly different (Figure 5.7). Similarly, the overall mRED Scores for embryos collected from the left uterine horn (0.94±0.05) and from the right uterine horn (0.87±0.04) was not significantly different. The mRED Score for male embryos
Table 5.2. Distribution of male and female embryos collected from the right and left uterine horns of superovulated beef cows.

<table>
<thead>
<tr>
<th>Uterine horn</th>
<th>No. of embryos</th>
<th>No. of male (%)</th>
<th>No. of female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>94</td>
<td>43 (45.7)&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>51 (54.3)&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Right</td>
<td>111</td>
<td>65 (58.6)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>46 (41.4)&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>205</td>
<td>108 (52.7)</td>
<td>97 (47.3)</td>
</tr>
</tbody>
</table>

<sup>a-b</sup>Numbers with different superscripts within a column are significantly different (P<0.05).
<sup>c-d</sup>Numbers with different superscripts within a row are significantly different (P<0.05).
<sup>*</sup>Numbers with an asterisk differ significantly from parity (P<0.05).
Figure 5.3. Percentage of embryos recovered from the left and right uterine horns of superovulated beef cows. \(^{a,b}\) Bars with different letters are significantly different (P<0.05). \(^*\) Bars with an asterisk differ significantly from parity (P<0.05).
Figure 5.4. Sex ratio (% male) of embryos recovered from the left and right uterine horns of superovulated beef cows. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 5.5. Distribution of male and female embryos recovered from the left and right uterine horns of superovulated beef cows. *a,b* Bars with different letters within each sex are significantly different (P<0.05). * Bars with an asterisk differ significantly from parity (P<0.05).
Figure 5.6  Sex ratio (% male) of embryos recovered from the left and right uterine horns of superovulated beef cows by replicate.  \(^a,b\) Bars with different letters within replicates are significantly different (P<0.05). \(^\ast\) Bars with an asterisk differ significantly from parity (P<0.05).
Table 5.3. Mean (±SEM) mRED Score for male and female embryos recovered from the left and right uterine horns of superovulated beef cows.

<table>
<thead>
<tr>
<th>Uterine horn</th>
<th>No. of embryos</th>
<th>Mean (±SEM) mRED Score Male</th>
<th>Mean (±SEM) mRED Score Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>94</td>
<td>0.94 (±0.09)</td>
<td>0.94 (±0.07)</td>
</tr>
<tr>
<td>Right</td>
<td>111</td>
<td>0.88 (±0.06)</td>
<td>0.85 (±0.07)</td>
</tr>
<tr>
<td>Total</td>
<td>205</td>
<td>0.91 (±0.05)</td>
<td>0.90 (±0.05)</td>
</tr>
</tbody>
</table>

*a-b Numbers with different superscripts within a column are significantly different (P<0.05).

c-d Numbers with different superscripts within a row are significantly different (P<0.05).
Figure 5.7. Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns superovulated beef cows. 

Bars with different letters are significantly different (P<0.05).
recovered from the left horn (0.94±0.09) was not significantly different from the mRED Score for female embryos from the same horn (0.94±0.07), and failed to differ from the mRED Score for male embryos collected from the right uterine horn (0.88±0.06). Likewise, the mRED Score for female embryos recovered from the right horn (0.85±0.07) was not significantly different from the mRED Score for male embryos from the same horn (0.88±0.06), and also failed to differ from the mRED Score for female embryos collected from the left uterine horn (0.94±0.07).

The overall mRED Score in Replicate 1 (0.95±0.05) was significantly higher than the overall mRED Score in Replicate 2 (0.84±0.05) (Figure 5.8). In addition, the mRED Score for male embryos in Replicate 1 differed significantly from the mRED Score for male embryos in Replicate 2 (0.97±0.07 vs. 0.81±0.07, respectively). However, the mRED Scores for female embryos did not differ between replicates.

**Discussion**

The results of this experiment compare favorably with previous reports in the scientific literature. Though a larger number of embryos were recovered from the right uterine horn compared with the left, the values were not significantly different. Previous observations in cattle have indicated that ovulations occur more frequently on the right ovary (Perkins et al., 1954; Aldahash and David, 1977; Hanrahan, 1983). However, in superovulated donors, reports of the number of CLs present on the left and right ovaries of donor females at embryo collection have been contradictory. Guilbault et al. (1991) and Cushman et al. (1999) found no difference in the number of CLs on the left and right ovaries of donor cattle, while Lauria et al. (1982) reported a significantly greater number of CLs present on the right ovary of Holstein heifers at the time of embryo collection.

The overall sex ratio of the embryos recovered in this study (52.7%) compares favorably with the reports of the sex ratio of calves produced from ET, though the deviation from parity was not statistically significant. A distortion of 2.7% would require at a minimum 820 observations to detect any significant difference (Moore and Gledhill, 1988). Unfortunately, this observation does not provide an answer to the origin of the higher-than-parity sex ratio of ET-derived calves. Perhaps a study with a greater number of observations will be able to identify the source of the distorted sex ratio reported in ET-derived calves if such a distortion exists.
Figure 5.8. Mean (±SEM) mRED Score of male and female embryos recovered from superovulated beef cows by replicate. a,b Bars with different letters between replicates are significantly different (P<0.05). c,d Bars with different letters between replicates are significantly different (P<0.05).
The altered sex ratios observed in calves gestated in the left and right uterine horns in previous experiments (Experiments 3.2 and 3.3) were also detected in the recovered embryos. While the sex ratio of the embryos collected from the left and right uterine horns in this experiment was not as distorted as the sex ratio of calves in Experiments 3.2 and 3.3, the difference was significant. However, the reason for the less severe distortion of the sex ratio remains unclear.

Gutierrez-Adan et al. (1999) and Park et al. (2005) hypothesized that the maturational state of the in vivo matured oocyte immediately prior to fertilization can influence the probability that either an X- or Y-chromosome bearing sperm will successfully fertilize the oocyte. Avery (1989) demonstrated that in superovulated cattle, ovulations occur over a prolonged period of time. This extended ovulation interval may produce COCs of various maturational states that partially mask the altered sex ratios found in the left and right uterine horns of single-ovulating cattle. Conversely, Insler and Lunenfeld (1996) hypothesized that an altered physiological state, induced by the administration of exogenous gonadotropins, may generate atypical oocytes compared with normal, untreated controls. This hypothesis supports the results of Callesen et al. (1986) and King et al. (1986), that described an increase in abnormal follicle development in addition to aberrant oocyte maturation in superovulated donors.

The time of insemination has been reported to influence the sex of the offspring in various species. In cattle, the timing of insemination relative to ovulation has resulted in distorted sex ratios (Wehner et al., 1997; Wells, 1980), though these reports remain controversial. Donors in the present experiment were inseminated multiple times, at 12 hours and 24 hours after the onset of estrus, based on electronic as well as visual estrus detection. In addition, ovulation has been reported to occur earlier after the onset of estrus in superovulated cattle (~28 hours) compared with unstimulated individuals (~32 hours) (Avery, 1989). This multiple insemination scheme may partially conceal the true sex ratio potential in the left and right horns, since inseminations occurred ~16 hours and ~4 hours prior to the onset of ovulation. Unfortunately, the effect of a single insemination, either early or late relative to ovulation, on the sex ratio of embryos recovered from donor cows remains unknown.
The development of male bovine embryos has been described to be more advanced compared with female embryos of the same chronological age (Avery et al., 1989; Thompson, 1997; Kochhar et al., 2001; Larson et al., 2001; Peippo et al., 2001). In cattle, this sex-dependent developmental asynchrony has been described in both in vivo (Gutierrez-Adan et al., 1999) and in vitro-derived (Xu et al., 1992; Tominaga et al., 1996) embryos. However, the results of this experiment cannot support this argument, as no difference in the mRED Scores of male and female embryos was detected. Again, this asynchronous development failure may be due to inferior oocyte maturational quality at the time of fertilization. Callesen et al. (1986) described an increase in abnormal follicle development, while Callesen et al. (1987) reported an increase in immature oocyte ovulations in superovulated cattle. In addition, an associated decrease in embryo quality was also described in both reports.
CHAPTER VI
SEX RATIO OF EMBRYOS RECOVERED FROM UNILATERALLY-OVARIECTOMIZED, SUPEROVULATED BEEF HEIFERS

Introduction
Interactive regulation of gonadal function through hormonal communication has been described in numerous mammalian species (Cunningham et al., 1978; Lacker, 1981; Hirshfield, 1991). For example, in the male, the remaining testis undergoes prompt compensatory hypertrophy following unilateral gonadectomy and sperm production eventually approaches the level present before removal of one gonad. This compensatory phenomenon has been described in the rat (Liang and Liang, 1970), mouse (Fawke et al., 1972) and hamster (Kishi et al., 2000), as well as in farm species, such as cattle (Johnson, 1978; Barnes et al., 1980a,b), sheep (Hochereau-de Reviers et al., 1976; Walton et al., 1978), horse (Gygax et al., 1973) and swine (Kosco et al., 1987; Minton and Wettemann, 1988).

Similarly, removal of a single ovary results in an acute compensatory increase in the number of large follicles present in the other ovary in multiple ovulating females, such as the rat (Pepper and Greenwald, 1970), pig (Brinkley et al., 1964), rabbit (Fleming et al., 1984) and hamster (Joshi and Labhsetwar, 1974; Greenwald and Bast, 1978). In cattle that have been unilaterally ovariectomized, this compensatory ovarian hypertrophy appears as an increase in the number of antral follicles that progress from small to medium size. Saiduddin et al. (1970) noted an increase in the number of follicles >5 mm in diameter in cows, while Johnson et al. (1985) and Lussier et al. (1987) reported an increase in the number of medium-size follicles following unilateral ovariectomy in heifers.

The response of unilaterally ovariectomized cows to superovulation has been related to the population of follicles present on the contralateral ovary at the time of removal (Cushman et al., 1999). Singh et al. (2004) described a simple ultrasound test to predict the response of donors to stimulation, indicating that the population of small and medium follicles on the ovaries at the emergence of a new follicular wave was positively correlated with the stimulatory response. Selection of the dominant follicle and ovulation may also be affected by unilateral ovariectomy. Mohan and Rajamahendran
(1998), using ultrasonography, demonstrated that unilateral ovariectomy in cattle resulted in sustained growth of the subordinate follicle and ovulation of multiple follicles ~75% of the time during the three subsequent estrous cycles.

However, a complete understanding of this lateral communication between the ovaries remains elusive and the role this interaction plays in regulating the sex ratio remains unknown. Therefore, the objective of the present study was to determine the sex ratio of embryos recovered from the left and right uterine horns of unilaterally ovariectomized superovulated beef heifers. The sex ratio of the embryos would provide insight into the role the ovary of origin has in influencing the sex of the embryos, as well as examine the effect interovary communications have in regulating the sex ratio of embryos derived from the left and right ovaries.

**Materials and Methods**

**Experimental Animals**

Crossbred beef heifers (n=20) were used in the present study to evaluate the sex ratio of embryos recovered from superovulated donors after unilateral ovariectomy. These animals were various crosses of Angus, Brahman, Brangus, Hereford and Simmental breeds and were categorized into two groups.

Heifers in Group A (n=10) were acquired from Idlewild Research Station (see Figure 3.2) located in Clinton, Louisiana. At the onset of this experiment, animals ranged in age from 20 to 22 months, while body weights ranged from 251.9 to 325.0 kg, with a mean (±SEM) of 287.2±8.1 kg. Conversely, Group B heifers (n=10) were obtained from Dean Lee Research Station (see Figure 3.2) located in Alexandria, Louisiana. Body weights for the heifers in Group B ranged from 453.6 to 549.5 kg with a mean (±SEM) of 502.9±12.6 kg with ages ranging from 32 to 34 months.

Body condition scores were assigned to each animal on a scale from 1 to 10 (1 = emaciated to 10 = obese). This condition score, modified from Wagner et al. (1988), was subjectively assigned to describe overall body condition, fat cover and flesh over the ribs, loin and tailhead. Body condition scores ranging from 6 to 8, based on visual appraisal and palpation of fat covering, are considered as optimal for normal reproductive function at this laboratory. Mean body condition scores for heifers in Group A and Group B were 5.3±0.2 and 7.4±0.2, respectively.
During this study, heifers from both groups were maintained in 0.8-hectare pasture lots. Forages available in pastures were established bermudagrass (*Cynodon dactylon*) and overseeded ryegrass (*Lolium multiflorum*). When forage was less than adequate, animals were supplemented with bermudagrass hay, while fresh water and mineral supplements were supplied *ad libidum*.

The St. Gabriel Research Station has a semitropical climate with a mean annual temperature of 19.7°C. January is the coldest month, with an average temperature of 5.4°C (minimum –5°C) while July and August are the warmest, with temperatures averaging 29°C (maximum 35°C). The average rainfall in this area is 155 cm per year. This experiment was conducted during the period from December, 2003, to April, 2004. During this period, nonsurgical embryo recoveries were performed on multiple donors on two separate dates (replicates).

**Experimental Design**

After a brief quarantine period upon arrival at St. Gabriel Research Station, five heifers each from Group A and Group B were randomly selected to undergo unilateral ovariectomy of the right ovary, while the left ovary was to remain intact (LOI). The remaining five animals in each group were designated to have the left ovary removed and the right ovary was to remain intact (ROI). Licensed veterinarians from the LSU School of Veterinary Medicine, using a flank approach, performed unilateral ovariectomies on all experimental animals. After the ovariectomy procedures were completed, a recovery period of ~45 days was allowed for all animals before the initiation of superovulatory treatment.

**Superovulation Protocol**

A timeline of the superovulatory protocol used for the superovulation of heifers in this experiment is illustrated in Figure 6.1. At the beginning of treatment, all heifers received a 2-ml intramuscular injection containing 25 mg of progesterone (P₄) and 1.25 mg of estradiol-17β (E₂) in sesame oil. At the same time, heifers received a controlled-release, intravaginal device (CIDR®) containing 1.38 g P₄ (EAZI-BREED®, DEC International, NZ, Ltd., Hamilton, New Zealand). A total of 28 mg of porcine FSH (Sioux Biochemical, Sioux Center, IA) was administered in seven intramuscular injections over 4 days using a descending-dose, twice-daily injection schedule beginning on the
Figure 6.1. Timeline of the superovulation protocol for unilaterally overiectomized beef heifers.
morning of Treatment day 5. Along with the fifth and sixth FSH injections, a 25-mg injection of PGF$_{2\alpha}$ (Lutalyse®, Upjohn Co., Kalamazoo, MI) was administered. The CIDR® was removed from all animals in the evening of Treatment day 7 concurrently with the final injection of PGF$_{2\alpha}$.

Experimental animals were observed twice daily, in the morning and evening, ~12 hours apart for estrual behavior. The estrual activity of all animals was also monitored with HeatWatch® system (DDx, Inc., Denver, CO). For this experiment, estrous parameters were defined as three mounts within a 2-hour period, with a minimum duration of 2 seconds per mount. Estrus was expected in the morning of Treatment day 9, though the onset of estrus in the evening of Treatment day 8 and in the evening of Treatment day 9 was considered acceptable. Donors were artificially inseminated at 12 and 24 hours after the onset of estrus, with 2 straws of semen from a Holstein bull of known fertility at each insemination. Embryos were nonsurgically recovered from each donor that exhibited estrus 7 days after the onset of estrus (estrus = day 0). Heifers that failed to exhibit standing estrus were not used in this experiment.

**Embryo Collection Procedure**

Nonsurgical embryo recoveries were performed on donors on day 7 following the onset of standing estrus, following a similar procedure to those reported by Drost et al. (1976), Elsden et al. (1976), Rowe et al. (1976) and Greve et al. (1977). Prior to embryo collection, females were restrained in a standard cattle squeeze chute and administered 15 mg of acepromazine (Prom Ace®, Fort Dodge Laboratories, Fort Dodge, IA). A 5-ml epidural injection of lidocaine (Lidocaine Hydrochloride 2%, Butler Columbus, OH) was administered into the first intercoccygeal space as a caudal epidural block. The ovaries of all animals were palpated *per rectum* and ultrasonically checked to determine the number of corpora lutea and follicles present on each ovary prior to embryo recovery procedure.

At the time of collection, an 18-French, two-way catheter (Cook Veterinary Products, Inc., Bloomington, IN) with stiffening stylette was guided through the cervix canal. Once the tip had penetrated the internal cervical os, the catheter was then slowly guided into uterine horn ipsilateral to the remaining intact ovary. Once the tip of the catheter was located at the greater curvature of the uterine horn, past the external
bifurcation, the cuff was inflated with 7 to 10 ml of flush medium to stabilize the uterine position and prevent back-flow. Embryo collection medium (Appendix A) was used to recovery embryos from the uterine horns in this experiment. The uterine horn was repeatedly flushed with 500 to 1,000 ml of medium. Embryo collection medium recovered from the uterus was filtered using a 75-µm, inline filter (EmCon®, Immuno Systems, Spring Valley, WI). Upon completion of the flushing procedure, the cuff was deflated and the catheter was slowly removed and drained into the filter.

The filtered flushing medium was then placed into 100 mm x 15 mm petri dishes (Falcon, Beckton-Dickinson & Co., Franklin Lakes, NJ) and maintained at room temperature (25°C). Embryo searching was performed by a separate technician using a stereomicroscope. Once visualized, embryos were transferred to embryo holding medium (Appendix B) by means of a capillary pipette (Unopette®, Becton Dickinson & Co., Rutherford, NJ).

Under phase-contrast magnification (100X) embryo quality (EQ) grades, ranging from 1 to 4 (1 = excellent to 4 = poorest quality), were subjectively assigned to each embryo, as previously characterized by Elsden et al. (1978) and Wright (1981). In this study, subjective grading of bovine embryos was based on symmetrical appearance of the embryo, presence of extruded blastomeres, degeneration of the cell mass and developmental synchrony to day of cycle of the donor, as described by Elsden et al. (1978) and Shea (1981). Ova were considered fertilized if the presence of cleavage divisions were apparent. Based on the developmental stage and quality grade, embryos were assigned a modified RED score (mRED) (Ryan et al., 1992). The mRED score was adapted from the Ryan Embryo Development score for use with in vitro cultured bovine embryos (see Table 5.1).

Prior to the onset of this study, two criteria were established for the inclusion of a heifer in the results of this experiment. First, each potential donor had to have exhibited standing estrus, as previously stated. Secondly, each heifer must have responded to FSH treatment, having two or more corpora lutea present at the time of embryo collection. Any donor not meeting these criteria was not included in the results summary. Recovered embryos were frozen for sex determination at a later date after grading and evaluation was completed.
Embryos to be frozen were placed in 1.4 M glycerol freezing medium (Appendix C), loaded into labeled 0.25 ml straws and held at room temperature for 10 minutes. The straws containing individual embryos were placed in a Planer R204 cell freezer (Planer Products, Ltd., Surrey, UK) maintained at -7°C with LN₂ vapor. Embryos were held at -7°C for 10 minutes. After holding at -7°C for 2 minutes, straws were seeded by touching supercooled forceps to the uppermost column of freezing medium. The embryos were subsequently cooled to -36°C at -0.5°C per minute. Upon completion of the freezing program, the straws were plunged into LN₂. Straws containing embryos were placed in labeled, 13-mm goblets, which were then attached to aluminum canes. Loaded canes were placed in dewar tanks containing LN₂ for storage until sexing.

At the time of thawing, frozen embryos were removed from the dewar, thawed in air for 10 seconds and thawed in a 30°C water bath for a additional 10 seconds. Upon expulsion from the straw, thawed embryos were immediately transferred to a 1M sucrose thawing medium (Appendix D) for a period of 15 to 20 minutes to allow rehydration. After rehydration, embryos were moved to a petri dish containing embryo holding medium (Appendix B) to await sex determination.

**Embryo Sex Determination**

Embryos were sexed using a modified protocol based on a commercially available bovine embryo sexing kit (AB Technology, Inc., Pullman, WA). The kit is designed to detect male specific, Y-chromosomal DNA sequences (Mathews et al., 1987; Bondioli et al., 1989) through the use of polymerase chain reaction (PCR). The kit was developed to amplify 8 to 10 trophectoderm cells obtained from biopsies based on the micromanipulation methodologies of Herr and Reed (1991). In the present experiment, the entire embryo was used in the PCR reaction to determine the sex, rather than 8 to 10 trophectoderm cells.

Individual embryos were repeatedly washed through phosphate-buffered saline containing 2 mg/ml of polyvinylpyrrolidone (PBS-PVP) to remove serum following rehydration. The zona pellucida of each embryo was removed by repeated, rapid pipetting of the embryo through a small-bore (~50 µm) glass pipette after a final washing to reduce the probability of DNA contamination. After the zona was removed, the embryo in 2 µl of PBS-PVP was placed in a 200-µl Eppendorf microcentrifuge tube.
containing 8 µl of ddH2O. The assay tubes containing the embryos were immediately plunged in LN2. The assay tubes were then held in an ice bath while additional embryos were processed. After a pool of 15 to 20 embryos had been processed, 12 µl of YCD® (Y-Chromosome Determinant) containing Taq polymerase, dNTP’s, male-specific (Y-chromosome) and nonmale specific (autosomal) probes, were added to each assay tube and thoroughly mixed. The reactions were amplified in a PTC-150 minicycler (MJ Research, Waltham, MA). The amplification profile of the thermocycler consisted of 1 minute at 95°C, followed by 33 cycles of 10 seconds at 95°C, 20 seconds at 64°C and 15 seconds at 72°C. Following completion of the final amplification cycle, assay tubes were held at 4°C.

After completion of thermocycling, 5 µl of gel loading buffer (Appendix E) was added to each assay tube and thoroughly mixed. Then 18 to 20 µl of the final PCR product was loaded into the designated wells of a 3% agarose gel (Appendix F) in a BioMax MP1015 electrophoresis unit (Kodak Imaging Systems, New Haven, CT). Electrophoresis of the gels was performed at 140 volts for 20 minutes. After electrophoresis, the gel was removed from the unit and the DNA bands were visualized by ethidium bromide fluorescence using ultraviolet illumination.

For each gel electrophoresis, a negative control, without embryonic or genomic DNA, was processed to detect possible contamination. Peripheral blood lymphocytes, serving as male and female controls, were also processed and run in each gel. The presence of a nonspecific DNA band indicated that a viable sample was present in the assay tube, while primer bands were occasionally visualized in lanes depending on the number of cells processed in that particular embryo. The presence of a male-specific band indicated male samples, while female samples were identified by the absence of this band under UV illumination (see Figure 5.2).

Statistical Analysis

Analysis of data in this experiment were performed utilizing the Glimmix Procedure (SAS, 2006) in SAS software Version 9.1.3 to analyze logistic regression models with fixed effects. The main effects and the differences between uterine horn sides were considered significant at the P<0.05 level. The total number of embryos and the sex of the embryos recovered from the uterine horns of unilaterally, ovariectomized
heifers, as well as the quality and stage of development of the recovered embryos, were used as the end points for side comparisons.

**Results**

The distribution of male and female embryos collected from the left and right uterine horns of unilaterally ovariectomized heifers is summarized in Table 6.1. A total of 53 embryos were collected and evaluated in this study. Of the 53 embryos, 42 (79.2%) were recovered from right-ovary intact heifers while 11 (20.8%) were recovered from left-ovary intact heifers (Figure 6.2). Significantly more embryos were recovered from right-ovary intact heifers compared with left-ovary intact heifers (P<0.05).

The overall sex ratio of the embryos recovered in this study was 41.5% and was not different from the expected sex ratio of 50% (Figure 6.3). In addition, the sex ratio of the embryos recovered from left-horn intact donors (45.5%) was not significantly different from the sex ratio of embryos recovered from the right-ovary intact donors (40.5%) and the sex ratio of the embryos recovered from both right- and left-ovary intact heifers failed to differ significantly from parity. The percentage of male and female embryos recovered from left- and right-ovary intact heifers is illustrated in Figure 6.4.

The sex ratio of embryos collected during each replicate is illustrated in Figure 6.5. The overall sex ratio of the embryos recovered in Replicate 1 (45.5%) and Replicate 2 (38.7%) were not significantly different. In addition, the sex ratio of embryos collected from right-ovary intact heifers were not significantly different from the sex ratio of embryos collected from left-ovary intact heifers in Replicate 1 (50.0% vs. 43.8%, respectively) or Replicate 2 (40.0% vs. 38.5%, respectively). The overall sex ratio, as well as the sex ratio of embryos from both left- and right-ovary intact heifers did not differ from parity in either replicate.

The sex ratio of embryos recovered from heifers in Group A and Group B is illustrated in Figure 6.6. The overall sex ratio of embryos from Group A heifers did not differ significantly from the sex ratio of embryos recovered from Group B heifers (43.6% vs. 35.7%, respectively). Additionally, the sex ratio of embryos recovered from left-ovary intact heifers in Group A (42.9%) was not significantly different from the sex ratio of
Table 6.1. Distribution of male and female embryos collected from the right and left uterine horns of unilaterally ovariectomized, superovulated beef heifers.

<table>
<thead>
<tr>
<th>Ovary intact</th>
<th>No. of embryos</th>
<th>No. of male (%)</th>
<th>No. of female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>11</td>
<td>5 (45.5)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td>Right</td>
<td>42</td>
<td>17 (40.5)</td>
<td>25 (59.5)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>22 (41.5)</td>
<td>31 (58.5)</td>
</tr>
</tbody>
</table>

\* Numbers with an asterisk differ significantly from parity (P<0.05).

| Numbers with different superscripts within a column are significantly different (P<0.05). |
| Numbers with different superscripts within a row are significantly different (P<0.05). |
Figure 6.2. Percentage of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers.  \(^{a,b}\)Bars with different letters are significantly different (P<0.05).  \(^\ast\)Bars with an asterisk differ significantly from parity (P<0.05).
Figure 6.3. Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 6.4. Percentage of male and female embryos collected from left- and right-ovary intact unilaterally ovariectomized beef heifers. Bars with different letters within intact ovaries are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 6.5. Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by replicate. *Bars with different letters within replicates are significantly different (P<0.05). *Bars with an asterisk differ significantly from parity (P<0.05).
Figure 6.6. Sex ratio (% male) of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by group. Bars with different letters within replicates are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05). No embryos were recovered from any right-ovary intact heifers in Group B.
embryos recovered from left-ovary intact heifers in Group B (35.7%). While the sex ratio of embryos recovered from right-ovary intact heifers in Group A was 45.5%, no embryos were recovered from right-ovary intact heifers in Group B.

The mean mRED Score values for male and female embryos collected from left- and right-ovary intact, superovulated heifers is summarized in Table 6.2. Overall, the mean mRED Score for all embryos collected in this experiment was 0.87±0.14 (Figure 6.7). In addition, the overall mRED Score for all embryos collected from right-ovary intact heifers was 0.61±0.15 while the overall mRED Score for all embryos collected from left-ovary intact heifers was 0.94±0.17. The overall mRED Score for all embryos collected from right- and left-ovary intact heifers was not significantly different. The overall mRED Score for male embryos collected from heifers was 0.61±0.15, while the overall mRED Score for female embryos was 0.94±0.17 (Figure 6.8). The overall mRED Score for male embryos and female embryos collected from all heifers was not significantly different.

The overall mRED Score in Replicate 1 (1.02±0.23) was not significantly different from the overall mRED Score in Replicate 2 (0.77±0.17) (Figure 6.9). In addition, the mRED Score for embryos recovered from left-ovary intact heifers in Replicate 1 was not significantly different from the mRED Score for embryos collected from left-ovary intact heifers in Replicate 2 (1.17±0.30 vs. 0.80±0.20, respectively). Similarly, the mRED Scores for embryos collected from right-ovary intact heifers did not differ between replicates (0.61±0.20 vs. 0.60±0.24, respectively).

The mRED Score for male embryos in Replicate 1 (0.90±0.49) was not significantly different from the overall mRED Score for male embryos in Replicate 2 (0.97±0.40) (Figure 6.10). Additionally, the mRED Score for female embryos in Replicate 1 (1.11±0.50) was not significantly different from the overall mRED Score for female embryos in Replicate 2 (0.64±0.28).

The mRED Scores of embryos recovered from heifers in Group A and Group B is illustrated in Figure 6.11. The overall mRED Score for embryos recovered from Group A heifers (0.99±0.28) was not significantly different from the overall mRED Score for embryos recovered from heifers in Group B (0.52±0.30). Similarly, the mRED Score for
Table 6.2. Mean (±SEM) mRED Score for male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers.

<table>
<thead>
<tr>
<th>Ovary</th>
<th>No. of embryos</th>
<th>Mean (±SEM) mRED Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Left</td>
<td>11</td>
<td>0.98 (±0.21)</td>
</tr>
<tr>
<td>Right</td>
<td>42</td>
<td>0.80 (±0.20)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>0.94 (±0.16)</td>
</tr>
</tbody>
</table>

\(^{a-b}\) Numbers with different superscripts within a column differ significantly (P<0.05).

\(^{c-d}\) Numbers with different superscripts within a row differ significantly (P<0.05).
Figure 6.7. Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers. Bars with different letters are significantly different (P<0.05).

Figure 6.7. Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers. Bars with different letters are significantly different (P<0.05).
<table>
<thead>
<tr>
<th>mRED Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.6</td>
</tr>
<tr>
<td>0.7</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>0.9</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.1</td>
</tr>
<tr>
<td>1.2</td>
</tr>
</tbody>
</table>

Figure 6.8. Mean (±SEM) mRED Score of male and female embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by sex.

Bars with different letters are significantly different (P<0.05).
Figure 6.9. Mean (±SEM) mRED Score of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by replicate. ^a^b^ Bars with different letters are significantly different (P<0.05).
Figure 6.10. Mean (±SEM) mRED Score of male and female embryos recovered from unilaterally ovariectomized beef heifers by replicate. Bars with different letters are significantly different (P<0.05).
Figure 6.11. Mean (±SEM) mRED Score of embryos recovered from the left and right uterine horns of unilaterally ovariectomized beef heifers by group. Bars with different letters are significantly different (P<0.05). ‡No embryos were recovered from any right-ovary intact heifers in Group B.
Figure 6.12. Mean (±SEM) mRED Score of male and female embryos recovered from unilaterally ovariectomized beef heifers by group.  \textsuperscript{a,b}Bars with different letters are significantly different (P<0.05).
embryos recovered from left-ovary intact heifers in Group A was not significantly different from the mRED Score for embryos collected from left-ovary intact heifers in Group B (1.14±0.43 vs. 0.52±0.30, respectively). While the mRED Score of embryos recovered from right-ovary intact heifers in Group A was 0.61±0.25, no embryos were recovered from right-ovary intact heifers in Group B.

The mRED Scores of male and female embryos recovered from heifers in Group A and Group B is illustrated in Figure 6.12. The overall mRED Score for male embryos recovered from Group A heifers (0.94±0.38) was not significantly different from the overall mRED Score for male embryos recovered from Group B heifers (0.93±0.52). Likewise, the mRED Score for female embryos recovered from heifers in Group A was not significantly different from the mRED Score for female embryos collected from heifers in Group B (1.03±0.37 vs. 0.30±0.22, respectively).

**Discussion**

Unfortunately, the results obtained in this experiment were less than ideal. The quantity of embryos recovered from the donors was insufficient to reveal any substantial insight into the effect of unilateral ovariectomy on the sex ratio. The reason for the poor response to superovulatory treatment remains unclear as no distinguishable pattern was detected in those animals that produced embryos and those that failed to respond to stimulation.

The pattern of the sex ratio of embryos recovered from left- and right ovary-intact females in this experiment (45.5% and 40.5%, respectively) was unlike that detected in the previous experiment. While the overall sex ratio of the recovered embryos (41.5%) was lower than the previously reported sex ratio of ET calves, the sex ratio was not significantly different from parity. In addition, the sex ratio of embryos from right-ovary intact heifers was numerically lower than those collected from left-ovary intact heifers. However, the sex ratios of the embryos recovered from the left-ovary and right-ovary intact heifers was not significantly different.

Unlike the pattern noted with the embryo sex ratios, the mRED Scores of embryos analyzed in this experiment compared favorably with previous reports of sex-specific embryo development. Numerically, the mRED Score for male embryos was greater compared with the mRED Score of female embryos. Male embryos have been
reported to develop faster than female embryos of the same chronological age in vivo (Gutierrez-Adan et al., 1999) and in vitro (Xu et al., 1992; Tominaga et al., 1996). However, the differences appearing in the mRED Scores of male and female embryos were likely not significantly different due to the limited number of embryos harvested from these donor females.

Further investigations into the effect of interovarian communications on the sex ratios of embryos derived from the left and right ovaries are warranted. Perhaps, in future studies, the use of mature cows of known fertility would supply a sufficient quantity of embryos to provide more meaningful results.
CHAPTER VII

SEX RATIO OF IN VITRO BOVINE EMBRYOS RESULTING FROM ABATTOIR-DERIVED OOCYTES ORIGINATING FROM THE LEFT AND RIGHT OVARIES

Introduction

The sex ratio of embryos produced following IVF is the subject of much controversy. This matter is further confounded by the fact that the sex ratio is not calculated at a common day of culture or developmental stage. In addition, the sex ratio of embryos is often inferred from the sex ratio of the calves following parturition. Hasler et al. (1995) reported that sex ratio of IVP calves did not differ significantly from parity. A male-biased sex ratio has been described in IVP embryos (Lonergan et al., 1999), while sex ratios not different from parity have also been reported (Carvalho et al., 1996; Gutierrez-Adan et al, 2001; Agung et al., 2005). To further complicate the analysis, culture conditions during maturation, fertilization and culture of bovine oocytes and embryos have also been implicated as a source of distorted sex ratios (Grisart et al., 1995; Dominko and First, 1997a,b; Pegoraro et al., 1998; Gutierrez-Adan et al, 1996, 2001). Bowman et al. (1998) postulated an oocyte-related “discouragement” of fertilization with sperm bearing either an X or Y chromosome may be partially responsible for the distorted sex ratios.

The maturational state of the oocyte at the time of insemination has been hypothesized to influence the sex ratio of the embryos produced following IVF (Jongbloet et al., 1996; Dominko and First, 1997a,b; Gutierrez-Adan et al, 1999; Park et al., 2005; Agung et al., 2006). However, there are few studies concerning the relationship between the in vitro maturation culture period of oocytes and the sex ratio of embryos and contradictory results are often reported. Agung et al. (2006) described a positive correlation between the sex ratio of bovine embryos and length of oocyte maturation. In contrast, Park et al. (2005) reported that a reduced time of oocyte maturation resulted in an increase in the proportion of male calves produced following embryo transfer.

Though reproductive lateral asymmetries have been described in numerous mammalian species, no reports are available describing the developmental potential of
oocytes derived from the left and right ovaries following oocyte maturation, fertilization and embryo culture in vitro. A laterally dimorphic maturation response may be responsible for the distorted sex ratios reported in calves and in vivo-produced embryos. This hypothesis is, however, difficult to test in vivo. Therefore, the objectives of the present study were 1) to evaluate the sex ratio of IVP embryos originating from oocytes derived from the left and right ovaries, and 2) to determine the effect of length of oocyte maturation period on the sex ratio of embryos produced from oocytes originating from the left and right ovaries.

**Materials and Methods**

**Experimental Design**

**Experiment 7.1**

The sex ratio of IVP embryos originating from oocytes derived from the left and right ovaries was investigated. Embryos were produced using a standard laboratory IVF protocol utilizing oocytes matured in vitro for 22 hours. Oocytes originating from the left and right ovaries, and the subsequently derived embryos, were maintained separately during maturation, fertilization and culture. For this study, IVF was performed on three separate dates (replicates) in April, 2004.

**Experiment 7.2**

The sex ratio of embryos developing from oocytes originating from the left and right ovaries and fertilized in vitro following different periods of maturation in vitro was examined. Following collection, immature oocytes were randomly allocated to one of five maturation treatment intervals. Oocytes assigned to Treatment 1 were matured in vitro for 18 hours (18 h) prior to fertilization. Likewise, oocytes assigned to Treatments 2, 3, 4 and 5 were matured in vitro for 20 hours (20 h), 22 hours (22 h), 24 hours (24 h) and 26 hours (26 h), respectively. Oocytes originating from the left and right ovaries and assigned to the various maturation treatments were maintained separately during maturation and fertilization. Following IVF, embryos produced from the oocytes from each maturation period were cultured separately and were evaluated on day 7 post-insemination. During the month of May, 2004, IVF was performed on four separate dates (replicates) to obtain data for this experiment.
Ovary Collection

On the day prior to collection, the abattoir was notified that ovaries would be obtained for experimental use. The abattoir processes dairy animals consisting primarily of Holstein cows as well as mixed-breed beef animals. The weight, age, breed composition and stage of reproductive cycle of the females varied depending on the day of ovary collections. However, the day of collection was based on when mature cows were being slaughtered, to assure the ovaries of immature heifers would not be used. At the abattoir, a single technician collected the ovaries from each animal, insuring that the left and right ovaries were properly identified and separation was maintained. Collected ovaries were placed in a plastic storage container that had previously been filled with Ovary Transport Medium (Appendix G).

After collection, ovaries were subjected to an additional cleansing with 500 ml of fresh Ovary Transport Medium, followed by wiping three times with ethanol-soaked gauze pads (4 x 4, Johnson and Johnson®, Arlington, TX). The ovaries were then transferred to a clean plastic bag containing fresh Ovary Transport Medium and held at 22 to 25°C during transport to the laboratory. Transport time for the ovaries ranged from 6 to 8 hours. Ovary transport time was calculated as the time from the collection of the first pair of ovaries to the time they arrived at the laboratory.

Oocyte Aspiration

Upon arrival at the laboratory, the ovaries were again washed with fresh Ovary Transport Medium and placed on a sterile drape. In an effort to prevent possible contamination, the ovaries were wiped an additional time with ethanol-soaked gauze. The ovaries were examined for structures that would indicate the stage of their reproductive cycle. Pre-ovulatory follicles were identified along with the presence of any CHs, CLs and CAs. All follicles ranging from 2 to 9 mm in diameter were aspirated using a 20-gauge, 37.5-mm needle (Kendall, Manfield, MA) attached to a 12-ml disposable luer syringe (Sherwood Davis and Geck, St. Louis, MO). Throughout the oocyte collection procedure, ovaries were repeatedly wiped with ethanol-soaked gauze pads to reduce possible contamination. Once all usable follicles were punctured, the aspirate was placed into sterile 15-ml plastic conical tubes (Corning, New York, NY) that were labeled with the appropriate aspiration time.
Maturation of Oocytes

Following follicle aspiration, the aspirate was dispensed into 100 mm x 15 mm Falcon® square style search dishes (Becton Dickinson & Co., Lincoln Park, NJ). The 15-ml conical tube was rinsed with 2 ml of Oocyte Holding Medium (Appendix H) to ensure that all oocytes were dislodged from the bottom and sides of the tube. A stereoscopic microscope (Nikon SMZ-2B, Tokyo, Japan) was used to visualize oocytes at which time the embryos were transferred using a 225-mm Pasteur pipette (Fisher Scientific™, Pittsburgh, PA) into a 35 mm x 10 mm Falcon® plastic Petri dish (Becton Dickinson & Co., Lincoln Park, NJ) containing 2 ml of Oocyte Holding Medium.

Oocytes were washed twice with Maturation Medium (Appendix I) before being placed into the final maturation droplets. After washing, 10 to 15 oocytes were randomly placed into 50-µl droplets of Maturation Medium covered with embryo-tested mineral oil (M-8410, Sigma Chemical Co., St. Louis, MO). After all oocytes were thoroughly washed and placed into maturation droplets, the dish was sealed in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and the internal atmosphere was charged with 90% N₂, 5% O₂ and 5% CO₂. After gassing, the modular incubator was placed in an incubator at 38°C in a humidified atmosphere of 5% CO₂ in air.

IVF Media Preparation

Prior to the removal of oocytes from maturation, IVF stock media were prepared. Brackett-Oliphant (BO) stocks, consisting of two parts (BO-A and BO-B), were prepared followed by CR1aa Stock Medium (Rosenkrans and First, 1994) (Appendices J, K and L, respectively). Following preparation of the BO stocks, Fertilization Medium (Appendix M), BO-Caffeine Medium (Appendix N), BSA-BO (0.6%) Medium (Appendix O) and BSA-BO (0.3%) Medium (Appendix P) were prepared.

Semen Preparation

After allowing for proper equilibration of the IVF media, the semen was prepared for the IVF procedure. First, an appropriately labeled 35 mm x 10 mm Falcon® plastic petri dish was placed into the incubator and allowed to warm to prevent temperature shock to the sperm. Then, a 37°C water bath was prepared for thawing of the semen.
Semen was obtained from a Holstein bull of known fertility. Frozen-thawed semen from this bull was used for IVF in all experiments. To thaw, the straw was removed from LN₂ and immediately placed in the water bath for 45 to 60 seconds. Then, the contents of the straw were expelled into a sterile 15-ml plastic conical tube. A total of 9 ml of pre-equilibrated BO-Caffeine Medium was added to the semen and the tube was centrifuged for 6 minutes at 200 x g. The supernatant was carefully removed, so as not to disrupt the pellet of sperm cells remaining at the bottom of the tube. An additional 9 ml of BO-Caffeine Medium was added and the contents of the tube were repeatedly pipetted to resuspend the pellet. The spermatozoa were then centrifuged again for 6 minutes at 200 x g. The supernatant was again removed, followed by the addition of 4 ml of BO-Caffeine Medium and 4 ml of BSA-BO (0.6%) Medium. The mixture was resuspended and the tube was placed in the incubator (38°C) for 20 minutes to allow equilibration.

**In Vitro Fertilization**

During sperm equilibration, oocytes were removed from maturation medium and placed into a 35 mm x 10 mm Falcon® plastic petri dish containing 2 ml of BSA-BO (0.3%) Medium. Oocytes were washed twice to remove excess Maturation Medium. Following washing, the oocytes were placed back into the incubator at 38°C during the preparation of fertilization droplets. The volume of the sperm sample was adjusted to achieve the appropriate concentration. Next, four 80-µl insemination droplets were placed in the previously labeled 35 mm x 10 mm Falcon® plastic petri dish and covered with mineral oil. Then, after 10 to 20 oocytes were placed into each insemination droplet, the dish was placed in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and the atmosphere was charged with 90% N₂, 5% O₂ and 5% CO₂ for 2 minutes. After charging, the modular incubator was placed in an incubator at 38°C in a humidified atmosphere of 5% CO₂ in air for 6 hours.

Prior to oocytes being removed from the insemination droplets, Oocyte Denuding Medium, Oocyte Wash Medium and IVC Medium (day 0 to 3) were prepared (Appendices Q, R and S, respectively), followed by preparation of 35 mm x 10 mm Falcon® plastic petri dishes for the fertilized ova. Oocyte Wash Medium was placed into
three petri dishes to await the next step of the procedure and all media were placed in a 5% CO₂ in air incubator to allow equilibration.

After the appropriate equilibration time, sperm-exposed ova were recovered from insemination droplets and transferred directly to the first dish of Oocyte Wash Medium. Cumulus-oocyte complexes were rinsed through the second dish of Oocyte Wash Medium to remove excess insemination medium, and were transferred into a 15-ml conical tube containing 1 ml of Oocyte Denuding Medium. The tube was then vortexed for 2 minutes to remove the remaining adherent cumulus cells.

Following vortexing, 2 ml of Oocyte Wash Medium was immediately added to the tube to stop the enzymatic activity of the hyaluronidase. The contents of the tube were then poured into an empty 35 mm x 10 mm Falcon® plastic petri dish, and the tube was rinsed with an additional 2 ml of Oocyte Wash Medium. Presumptive zygotes were recovered and transferred into the final dish of Oocyte Wash Medium and placed in an incubator at 38°C in a humidified atmosphere of 5% CO₂ in air during the preparation of the culture droplets.

In Vitro Culture

An empty petri dish was labeled with the respective treatment, the time and the date, followed by the addition of four 50-µl droplets of IVC Medium with an overlay of mineral oil. Then, 10 to 15 embryos were transferred to each culture droplet and the petri dish was placed in a modulator incubator chamber. The modular incubator was purged with a mixture of 5% CO₂, 5% O₂ and 90% N₂ at 1.4 kg/cm² (equivalent to 20 psi) for 2 minutes. The chamber was sealed and placed in an incubator maintained at 38°C and 5% CO₂ in air. Embryo development was evaluated on day 3 post-insemination (day 0 = oocytes placed in insemination droplets).

Prior to the evaluation of embryo development, IVC Medium (day 3 to day 7) (Appendix T) was prepared and allowed to equilibrate for 20 minutes in the incubator. Embryonic development was recorded and medium was changed after 3 days of incubation. Embryos were assessed for cleavage patterns and evidence of degeneration. The total number of 2-cell, 4-cell, 6 to 8-cell and 16-cell embryos was recorded. In addition, embryos that appeared to have degenerated in culture were recorded and transferred to a separate droplet. Embryos were transferred to a 35 mm x
10 mm Falcon® plastic petri dish containing 2 ml of IVC Medium (day 3 to day 7) and were washed two times to remove excess medium and by-products generated during the culture period.

Developing embryos (n=10 to 15) were transferred into 50-µl droplets of fresh IVC Medium (day 3 to day 7) covered with mineral oil. Petri dishes were then placed back into the airtight modulator incubator and charged for 2 minutes with a 5% CO₂, 5% O₂ and 90% N₂ gas mixture. The chamber was placed in an incubator at 38°C in a humidified atmosphere of 5% CO₂ in air and embryos were allowed to develop until day 7 post-insemination.

**Embryo Development**

On day 7 post-insemination, the developmental stage and quality of all embryos was documented. Embryo quality (EQ) grades, ranging from 1 to 4 (1 = excellent to 4 = poorest quality), were subjectively assigned to each embryo. In this study, subjective grading of bovine embryos was based on symmetrical appearance of the embryo, presence of extruded blastomeres, degeneration of the cell mass and developmental synchrony to day of culture. In addition, embryos were assigned a modified RED score (mRED) based on the developmental stage and quality grade. The mRED score was adapted from the Ryan Embryo Development score as reported by Ryan et al. (1992) for use with in vitro culture of bovine embryos (see Table 5.1). After embryo evaluation was completed, embryos were cryopreserved for sexing at a later date.

Embryos to be frozen were placed in 1.4 M glycerol freezing medium (Appendix C), loaded into labeled 0.25 ml plastic straws and held a room temperature for 10 minutes. The straws containing individual embryos were placed into a Planer R204 cell freezer (Planer Products, Ltd., Surrey, UK) maintained at -7°C with LN₂ vapor. Embryos were seeded by touching supercooled forceps to the uppermost column of freezing medium after being held at -7°C for 2 minutes, then held for an additional 8 minutes at -7°C. The embryos were subsequently cooled to -36°C at -0.5°C per minute. Upon completion of the freezing program, the straws were plunged into LN₂. Groups of straws containing embryos were placed in labeled goblets, which were then placed on aluminum canes and placed in dewar tanks containing LN₂ for storage until the sexing procedure.
At the time of thawing, frozen embryos were removed from the dewar, thawed in air for 10 seconds and thawed in a 30°C water bath for an additional 10 seconds. Thawed embryos were placed in 1M sucrose thaw medium (Appendix D) for a period of 15 minutes to allow rehydration. After rehydration, embryos were placed in holding medium (Appendix B) to await sexing.

**Embryo Sex Determination**

Embryos were sexed using a modified protocol based on the bovine embryo sexing kit supplied by AB Technology (AB Technology, Inc., Pullman, WA). The kit, through the use of polymerase chain reaction (PCR), is designed to detect male specific, Y-chromosomal DNA sequences (Mathews et al., 1987; Bondioli et al., 1989) and is based on the micromanipulation methodologies of Herr and Reed (1991). In the present experiment, the entire embryo was used in the PCR reaction rather than a biopsy of 8 to 10 cells removed from the trophectoderm.

Following rehydration, individual embryos were washed three times through phosphate-buffered saline containing 2 mg/ml polyvinylpyrrolidone (PBS-PVP) to remove serum. In an effort to reduce the probability of DNA contamination, the zona pellucida of each embryo was removed by repeated, rapid pipetting of the embryo through a small-bore (~50 µm) glass pipette after the final washing. Following zona removal, the embryo in 2 µl of PBS-PVP was placed in a 200-µl Eppendorf microcentrifuge tube containing 8 µl of ddH2O. After addition of the embryo, the assay tubes were immediately plunged in LN2, then held in an ice bath while additional embryos were processed.

Once a pool of 15 to 20 embryos had been processed, 12 µl of YCD® (Y-Chromosome Determinant) containing Taq polymerase, dNTP’s, male-specific (Y-chromosome) and nonmale specific (autosomal) probes, were added to the assay tube and thoroughly mixed. The reactions were amplified in a PTC-150 minicycler (MJ Research, Waltham, MA). The temperature profile consisted of 1 minute at 95°C, followed by 33 cycles of 10 seconds at 95°C, 20 seconds at 64°C and 15 seconds at 72°C and held at 4°C following completion of the last temperature cycle.

Following amplification, 5 µl of gel loading buffer (Appendix E) was mixed with the contents of each assay tube, and 18 to 20 µl of the PCR product was loaded into the
designated wells of a 3% agarose gel (Appendix F) in a BioMax MP1015 electrophoresis unit (Kodak Imaging Systems, New Haven, CT). Electrophoresis of the gels was run at 140 volts for 20 minutes. DNA bands were visualized by ethidium bromide fluorescence using ultraviolet illumination.

For each gel electrophoresis, a negative control to detect contamination consisting of a sample without embryo or genomic DNA was processed. Peripheral blood lymphocytes, serving as male and female controls, were also processed and included in each gel. The presence of a nonspecific DNA band indicated that a viable sample was present in the assay tube. A primer band was occasionally visible depending on the number of cells in the embryo. The presence of a male-specific band identified male samples, while female samples were identified by the absence of this band under UV illumination. A representative gel under UV illumination after electrophoresis is illustrated in Figure 5.2.

Statistical Analysis

Data in this experiment were processed using SAS software Version 9.1.3 and analysis was performed using the Glimmix Procedure (SAS, 2006) to evaluate logistic regression models with fixed effects. The main effects and the differences between the ovaries of origin were considered significantly different at the P<0.05 level. The total number of embryos and the sex of the embryos derived from oocytes originating from the left and right ovaries, as well as the quality and developmental stage of the embryos, were used as the end points for side comparisons.

Results

Experiment 7.1

The distribution of male and female embryos produced from oocytes originating from the left and right ovaries is summarized in Table 7.1. Of a total of 447 IVF-derived embryos, 239 (53.5%) were produced from left-ovary oocytes, while 208 (46.5%) were produced from oocytes from the right ovary.

The overall sex ratio of the embryos produced in this study was 43.4% and was significantly lower than the expected sex ratio of 50% (Figure 7.1). However, the sex ratio of the embryos produced from left-ovary oocytes (44.8%) was not significantly different from the sex ratio of embryos derived from the right-ovary oocytes (41.8%). In
Table 7.1. Distribution of male and female embryos originating from oocytes derived from the left and right ovaries.

<table>
<thead>
<tr>
<th>Oocyte source</th>
<th>No. of embryos</th>
<th>No. of male (%)</th>
<th>No. of female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>239</td>
<td>107 (44.8)</td>
<td>132 (55.2)</td>
</tr>
<tr>
<td>Right</td>
<td>208</td>
<td>87 (41.8)</td>
<td>121 (58.2)</td>
</tr>
<tr>
<td>Total</td>
<td>447</td>
<td>194 (43.4)*</td>
<td>253 (56.6)*</td>
</tr>
</tbody>
</table>

* Numbers with an asterisk differ significantly from parity (P<0.05).

\(^{a-b}\)Numbers with different superscripts within a column are significantly different (P<0.05).

\(^{c-d}\)Numbers with different superscripts within a row are significantly different (P<0.05).
Figure 7.1. Sex ratio (% male) of embryos derived from oocytes originating from the left and right ovaries. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
addition, the sex ratio of the embryos produced from both left-ovary and right-ovary derived oocytes were not significantly different from parity (P=0.06 and P=0.08, respectively). The percentage of male and female embryos produced from left-ovary and right-ovary oocytes is illustrated in Figure 7.2.

The sex ratio of embryos produced during each IVF replicate is illustrated in Figure 7.3. The overall sex ratio of the embryos in Replicate 1 (44.0%), Replicate 2 (44.4%) and Replicate 3 (42.6%) was not significantly different. In addition, the sex ratio of embryos from right-ovary oocytes did not differ significantly from the sex ratio of embryos produced from left-ovary oocytes in Replicate 1 (48.0% vs. 39.7%, respectively), Replicate 2 (50.0% vs. 37.5%, respectively) or Replicate 3 (40.5% vs. 45.0%, respectively). The overall sex ratio of embryos produced in each replicate did not differ from parity. Though the sex ratio of embryos produced from left- and right-ovary oocytes failed to differ from parity in Replicates 1 and 2, the sex ratio of embryos originating from the right ovary was significantly lower than parity in Replicate 3 (P=0.048). However, in the same replicate, the sex ratio of left-ovary derived embryos was not different from parity.

The mean mRED Score values for male and female embryos produced from left-ovary and right-ovary derived oocytes are summarized in Table 7.2. Overall, the mean mRED Score for all embryos produced in this experiment was 1.20±0.07 (Figure 7.4). In addition, the overall mRED Score for all embryos originating from left-ovary oocytes was 1.16±0.10 while the overall mRED Score for all embryos from right-ovary derived oocytes was 1.23±0.10. The overall mRED Score for all embryos produced from the left and right ovaries did not differ significantly. The overall mRED Score for male embryos produced in this experiment was 1.25±0.11 while the overall mRED Score for female embryos was 1.16±0.09. The overall mRED Scores for male and female embryos produced were not significantly different.

In addition, the mRED Score for male embryos originating from left-ovary oocytes (1.14±0.16) was not significantly different from the mRED Score for female embryos from the same ovary (1.18±0.13), and also failed to differ from the mRED Score for male embryos from the right ovary (1.33±0.15). Likewise, the mRED Score for female
Figure 7.2. Percentage of male and female embryos derived from oocytes originating from the left and right ovaries. Bars with different letters within each sex are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 7.3. Sex ratio (% male) by replicate of embryos derived from oocytes originating from the left and right ovaries. Bars with different letters within IVF-derived replicates are significantly different (P<0.05). Bars with different letters between replicates are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Table 7.2. Mean (±SEM) mRED Score for male and female in vitro-derived embryos originating from the left and right ovaries.

<table>
<thead>
<tr>
<th>Ovary of origin</th>
<th>No. of embryos</th>
<th>Mean (±SEM) mRED Score</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>239</td>
<td>1.14 (±0.16)</td>
<td>1.18 (±0.13)</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>208</td>
<td>1.33 (±0.15)</td>
<td>1.15 (±0.13)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>447</td>
<td>1.25 (±0.11)</td>
<td>1.16 (±0.09)</td>
<td></td>
</tr>
</tbody>
</table>

^ab^ Numbers with different superscripts within a column differ significantly (P<0.05).
^cd^ Numbers with different superscripts within a row differ significantly (P<0.05).
Figure 7.4. Mean (±SEM) mRED Score of male and female embryos derived from oocytes originating from the left and right ovaries. Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
embryos produced from right-ovary derived oocytes (1.15±0.13) was not significantly different from the mRED Score for male embryos derived from the same ovary (1.33±0.15), and failed to differ significantly from the mRED Score for female embryos produced from oocytes originating from the left ovary (1.18±0.13).

The overall mRED Score for embryos produced in Replicate 1 (1.07±0.12) was not significantly different from the mRED Score for embryos in Replicate 2 (1.02±0.15) (Figure 7.5). However, the overall mRED Score for embryos in Replicate 3 (1.36±0.10) was significantly higher than the mRED Scores in both Replicate 1 and Replicate 2. In addition, the mRED Scores for male embryos in Replicate 1 (1.11±0.19), Replicate 2 (1.08±0.24) and Replicate 3 (1.40±0.18) were not significantly different. Additionally, the mRED Score for female embryos in Replicate 1 (1.04±0.15) failed to differ significantly from the mRED Score of female embryos in Replicates 2 and 3 (0.97±0.19 and 1.32±0.14, respectively). However, the mRED Score for female embryos produced in Replicate 3 was significantly higher than the mRED Score of embryos of the same sex in Replicate 2.

The mRED Score of embryos produced from oocytes derived from the left and right ovaries during each replicate is illustrated in Figure 7.6. The mRED Score for embryos produced from left-ovary oocytes in Replicate 1 (1.07±0.17) failed to differ significantly from the mRED Score for left-ovary embryos produced in Replicate 2 and Replicate 3 (0.92±0.21 and 1.33±0.15, respectively). Similarly, the mRED Score for embryos from right-ovary oocytes did not differ between replicates (1.08±0.17, 1.09±0.20 and 1.38±0.14, respectively). However, the mRED Score for left-ovary embryos in Replicate 2 (0.92±0.21) was significantly different compared with the mRED Score of embryos from both the left and right ovary in Replicate 3 (1.33±0.15 and 1.38±0.14, respectively).

**Experiment 7.2**

The distribution of male and female embryos produced from oocytes originating from the left and right ovaries and matured for different time periods is summarized in Table 7.3. A total of 407 bovine embryos were produced and evaluated in this
Figure 7.5. Mean (±SEM) mRED Score of male and female in vitro-produced embryos by replicate.  
th
Overall (both sexes) mean bars with different letters are significantly different (P<0.05).  
th
Male and female bars with different letters are significantly different (P<0.05).
Figure 7.6. Mean (±SEM) mRED Score of embryos derived from oocytes originating from the left and right ovaries by replicate. a,b Overall (both ovaries) bars with different letters are significantly different (P<0.05). c,d Left and right bars with different letters are significantly different (P<0.05).
Table 7.3. Distribution of male and female embryos originating from oocytes derived from the left and right ovaries after different time periods of in vitro maturation.

<table>
<thead>
<tr>
<th>Trt</th>
<th>Left</th>
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<th>Right</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>No. of male (%)</td>
<td>No. of female (%)</td>
<td>n</td>
</tr>
<tr>
<td>18 h</td>
<td>34</td>
<td>17 (50.0)</td>
<td>17 (50.0)</td>
<td>45</td>
</tr>
<tr>
<td>20 h</td>
<td>26</td>
<td>12 (46.2)</td>
<td>14 (53.8)</td>
<td>59</td>
</tr>
<tr>
<td>22 h</td>
<td>53</td>
<td>24 (45.3)</td>
<td>29 (54.7)</td>
<td>49</td>
</tr>
<tr>
<td>24 h</td>
<td>36</td>
<td>15 (41.7)</td>
<td>21 (58.3)</td>
<td>35</td>
</tr>
<tr>
<td>26 h</td>
<td>44</td>
<td>18 (40.9)</td>
<td>26 (59.1)</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>193</td>
<td>86 (44.6)</td>
<td>107 (55.4)</td>
<td>214</td>
</tr>
</tbody>
</table>

*a,b* Numbers with different superscripts within a column are significantly different (P<0.05).

*c,d* Numbers with different superscripts within a row are significantly different (P<0.05).

*Numbers with an asterisk differ significantly from parity (P<0.05).*
experiment. Of the 407 embryos produced, 214 (52.6%) were derived from left-ovary oocytes and 193 (47.4%) were produced from oocytes from the right ovary.

The overall sex ratio of the embryos produced in this study was 45.9% and was not significantly different from the expected sex ratio of 50% (Figure 7.7). Similarly, the sex ratio of the embryos produced from left-ovary oocytes (44.6%) was not significantly different from the sex ratio of embryos derived from the right-ovary oocytes (47.2%) and the sex ratio of the embryos produced from both left-ovary and right-ovary derived oocytes was not significantly different from parity. The percentage of male and female embryos produced from oocytes derived from the left and right ovaries is illustrated in Figure 7.8.

The sex ratio of embryos produced after different maturation periods is shown in Figure 7.9. The overall sex ratio of embryos produced after 18 hours (50.6%) of oocyte maturation did not differ significantly compared with 20 hours (41.2%), 22 hours (52.0%) or 26 hours (45.7%) of oocyte maturation. In contrast, the sex ratio of embryos after 24 hours of maturation (38.0%) was significantly lower than the sex ratio of embryos produced after 18 and 22 hours of maturation (50.6% and 52.0%, respectively), but was not different than 20 and 26 hours of maturation (41.2% and 45.7%, respectively). The overall sex ratio of embryos produced in each oocyte maturation time period was not significantly different from parity.

The sex ratio of embryos produced from oocytes originating from the left and right ovaries during each IVF replicate is illustrated in Figure 7.10. The overall sex ratio of the embryos in Replicate 1 (47.1%), Replicate 2 (46.1%) and Replicate 4 (51.5%) was not significantly different. However, the overall sex ratio of embryos produced in Replicate 3 (39.2%) was significantly lower than Replicate 4 (51.5%), but was significantly different when compared with Replicate 1 and Replicate 2 (47.1% and 46.1%, respectively). The overall sex ratio of embryos produced in each replicate was not significantly different from the expected sex ratio of 50%. The sex ratio of embryos produced from oocytes originating from the left and right ovaries were not significantly different in Replicate 1 (49.2% and 44.2%, respectively), Replicate 2 (46.8% and 45.5%, respectively) and Replicate 4 (43.2% and 57.9%, respectively). In contrast, the sex ratio of embryos produced from oocytes originating from the left ovary in Replicate 3 (37.2%)
Figure 7.7. Overall sex ratio (% male) of embryos originating from oocytes derived from the left and right ovaries after in vitro maturation. *Bars with different letters are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 7.8. Distribution of male and female embryos derived from oocytes originating from the left and right ovaries after in vitro maturation. Bars with different letters within each sex are significantly different (P<0.05). Bars with an asterisk differ significantly from parity (P<0.05).
Figure 7.9. Sex ratio (% male) by treatment of embryos derived from oocytes originating from the left and right ovaries after different time periods of in vitro maturation. \(^{a,b}\) Overall bars with different letters within treatments are significantly different (P<0.05). \(^{c,d}\) Bars for left and right ovaries with different letters within treatments are significantly different (P<0.05). \(^*\) Bars with an asterisk are significantly different from parity (P<0.05).
Figure 7.10. Sex ratio (% male) by replicate of embryos derived from oocytes originating from the left and right ovaries after in vitro maturation. 

Overall bars with different letters within replicates are significantly different ($P<0.05$). Bars for left and right ovaries with different letters within replicates are significantly different ($P<0.05$).

Bars with an asterisk differ significantly from parity ($P<0.05$).
was significantly lower than the sex ratio of right-ovary derived embryos produced in Replicate 4 (57.9%). The sex ratio of right-ovary embryos in Replicate 3 (40.7%) was not different than the sex ratio of embryos produced from oocytes originating from either the left or right ovary in Replicates 1, 2 or 4. Similarly, the sex ratio of embryos produced from oocytes originating from the left and right ovaries was not significantly different from parity in each replicate.

The sex ratio of embryos produced after each maturation period in Replicates 1 through 4 is shown in Figure 7.11. The sex ratio of embryos produced after 18 hours of oocyte maturation did not differ significantly among Replicate 1 (40.0%), Replicate 2 (45.0%), Replicate 3 (55.0%) or Replicate 4 (63.2%). In addition, the sex ratio of embryos produced after 20 hours and 22 hours of oocyte maturation was not significantly different when compared with the same maturation periods in Replicate 1 (38.1% and 61.5%, respectively), Replicate 2 (47.6% and 50.0%, respectively), Replicate 3 (33.3% and 48.0%, respectively) or Replicate 4 (45.5% and 48.0%, respectively).

Similarly, the sex ratio of embryos produced from oocytes matured for 24 hours and 26 hours was not significantly different when compared with the same respective time periods in Replicate 1 (55.5% and 35.3%, respectively), Replicate 2 (29.4% and 55.5%, respectively), Replicate 3 (22.2% and 33.3%, respectively) or Replicate 4 (44.4% and 58.8%, respectively). Likewise, no significant differences in embryo sex ratio were detected when maturation periods were compared within and between replicates and the sex ratio of embryos produced in each oocyte maturation time period did not differ from parity in all replicates.

The mean mRED Score values for male and female embryos produced from left-ovary and right-ovary derived oocytes matured for different periods of time are summarized in Table 7.4. Overall, the mean mRED Score for all embryos produced in this experiment was 1.27±0.08 (Figure 7.12). In addition, the overall mRED Score for all embryos originating from left-ovary oocytes was 1.32±0.11 while the overall mRED Score for all embryos from right-ovary derived oocytes was 1.22±0.10. The overall mRED Score for all embryos produced from the left and right ovaries was not significantly different. The overall mRED Score for male embryos produced in this
Figure 7.11. Sex ratio (% male) by replicate of embryos after different treatment periods of in vitro maturation. a,b Bars with different letters are significantly different (P<0.05). *Bars with an asterisk differ significantly from parity (P<0.05).
Table 7.4. Mean (±SEM) mRED Scores of male and female embryos originating from oocytes derived from the left and right ovaries after different periods of in vitro oocyte maturation.

<table>
<thead>
<tr>
<th>Trt</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>18 h</td>
<td>0.93 (±0.33)(^{a,c})</td>
<td>0.98 (±0.31)(^{a,c})</td>
<td>0.96 (±0.27)(^{a,c})</td>
<td>1.21 (±0.36)(^{a,b,c})</td>
</tr>
<tr>
<td>20 h</td>
<td>2.39 (±0.49)(^{b,c})</td>
<td>0.83 (±0.31)(^{a,d})</td>
<td>1.31 (±0.32)(^{a,b,c,d})</td>
<td>1.29 (±0.26)(^{a,d})</td>
</tr>
<tr>
<td>22 h</td>
<td>1.67 (±0.37)(^{b,c})</td>
<td>1.71 (±0.34)(^{b,c})</td>
<td>1.26 (±0.28)(^{a,b,c,d})</td>
<td>0.74 (±0.25)(^{b,d})</td>
</tr>
<tr>
<td>24 h</td>
<td>1.25 (±0.42)(^{a,b,c})</td>
<td>1.19 (±0.36)(^{a,b,c})</td>
<td>1.27 (±0.43)(^{a,b,c})</td>
<td>1.32 (±0.32)(^{a,c})</td>
</tr>
<tr>
<td>26 h</td>
<td>0.94 (±0.32)(^{a,c})</td>
<td>1.24 (±0.30)(^{a,b,c,d})</td>
<td>1.90 (±0.45)(^{b,d})</td>
<td>1.03 (±0.37)(^{a,b,c,d})</td>
</tr>
<tr>
<td>Total</td>
<td>1.40 (±0.18)(^{c})</td>
<td>1.27 (±0.15)(^{c})</td>
<td>1.29 (±0.15)(^{c})</td>
<td>1.15 (±0.14)(^{c})</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Numbers with different superscripts within a column are significantly different (P<0.05).

\(^{c,d}\)Numbers with different superscripts within a row are significantly different (P<0.05).
Figure 7.12. Mean (±SEM) mRED Scores of male and female embryos derived from oocytes originating from the left and right ovaries after all time periods of in vitro maturation. a,bBars with different letters are significantly different (P<0.05).
The overall mRED Score of embryos originating from the left and right ovaries after different maturation periods is illustrated in Figure 7.13. The overall mRED Score of embryos produced after 18 hours of oocyte maturation (1.02±0.16) was significantly lower compared with 20 hours (1.37±0.17) and 22 hours (1.38±0.16) of maturation but failed to differ from maturation for 24 hours (1.126±0.18) and 26 hours (1.26±0.18). No difference in overall embryo mRED Score was observed between 20, 22, 24 and 26 hours of maturation. The mRED Score for embryos originating from the left ovary after 18 hours of maturation (0.95±0.22) was significantly lower compared with left-ovary derived embryos after 20 hours (1.55±0.31) and 22 hours (1.69±0.25) of oocyte maturation but was not significantly different from left-ovary derived embryos originating from oocytes matured for 24 hours (1.22±0.27) and 26 hours (1.12±0.22). Mean embryo mRED Scores for left-ovary derived embryos after 20, 22, 24 and 26 hours of maturation were not significantly different.

In addition, the mean mRED Score for left-ovary derived embryos after 18 hours of maturation (0.95±0.22) was not significantly different compared with right-ovary derived embryos after 18, 20, 22, 24 or 26 hours oocyte maturation (1.08±0.22, 1.30±0.20, 1.05±0.20, 1.30±0.25 and 1.50±0.30, respectively). However, the mRED Score of embryos originating from right-ovary derived oocytes matured for 22 hours (1.05±0.20) differed significantly compared with left-ovary derived embryos from oocytes matured for 20 hours (1.55±0.31) and 22 hours (1.69±0.25). Conversely, the mean
Figure 7.13. Mean (±SEM) mRED Scores of embryos originating from oocytes derived from the left and right ovaries after different time periods of in vitro maturation. 

a,b Overall bars with different letters are significantly different (P<0.05). c,d Bars for left and right ovaries with different letters are significantly different (P<0.05).
mRED Scores for embryos originating from the right ovary was not significantly different among in vitro maturation periods.

The mean mRED Scores of male and female embryos produced after different periods of in vitro maturation is displayed in Figure 7.14. The mRED Scores of female embryos was not significantly different among 18 hours (1.11±0.24), 20 hours (1.16±0.21), 22 hours (1.31±0.23), 24 hours (1.26±0.24) and 26 hours (1.18±0.24) of oocyte maturation. In addition, the mRED Scores of female embryos from all maturation periods also was not significantly different when compared with male embryos matured for 18, 20, 22, 24 and 26 hours (0.94±0.20, 1.68±0.28, 1.45±0.23, 1.26±0.30 and 1.37±0.27, respectively). In contrast, the mRED Score of male embryos derived from oocytes matured for 18 hours (0.94±0.20) was significantly lower than for male embryos from oocytes matured for 20 (1.68±0.28) and 22 (1.45±0.23) hours. However, the mRED Scores of male embryos originating from oocytes matured for 20, 22, 24 and 26 hours were not significantly different.

The mRED Scores of embryos produced from oocytes derived from the left and right ovaries during each replicate are illustrated in Figure 7.15. The overall mRED Score for embryos produced in Replicate 1 (1.35±0.15), Replicate 2 (1.44±0.17) and Replicate 3 (1.31±0.14) failed to differ significantly. However, the overall mRED Score for embryos produced in Replicate 1, Replicate 2 and Replicate 3 were significantly higher compared with the overall mRED Score in Replicate 4 (0.98±0.15). The mRED Score for embryos from right-ovary oocytes did not differ between replicates (1.11±0.19, 1.41±0.23, 1.27±0.19 and 1.07±0.20).

In addition, the mRED Score for left-ovary derived embryos in Replicate 1 (1.52±0.22), Replicate 2 (1.47±0.25) and Replicate 3 (1.37±0.22) were not significantly different. However, the mRED Score for embryos originating from left-ovary derived oocytes in Replicate 4 (0.87±0.22) was significantly lower compared with embryos from the same side in Replicate 1, but not Replicates 2 and 3. The mRED Score for embryos originating from right-ovary oocytes in Replicate 4 (1.07±0.20) was significantly lower compared with left-ovary derived embryos in Replicate 1(1.52±0.22), but were not
Figure 7.14. Mean (±SEM) mRED Scores of male and female embryos after different time periods of in vitro maturation. Bars with different letters are significantly different (P<0.05).
Figure 7.15. Overall mean (±SEM) mRED Scores by replicate of embryos originating from the left and right ovaries after all time periods of in vitro maturation. \(^{a,b}\)Overall bars with different letters are significantly different (P<0.05). \(^{c,d}\)Bars for left and right ovaries with different letters are significantly different (P<0.05).
different when compared with left-ovary derived embryos in Replicate 2 (1.47±0.25) and Replicate 3 (1.37±0.22).

The mRED Scores of male and female embryos produced in each replicate is shown in Figure 7.16. The mRED Scores for female embryos in Replicate 1 (1.38±0.22), Replicate 2 (1.12±0.20), Replicate 3 (1.29±0.18) and Replicate 4 (1.04±0.22) were not significantly different. Furthermore, the mRED Scores for male embryos in Replicate 1 (1.30±0.21), Replicate 2 (1.82±0.27) and Replicate 3 (1.35±0.22) failed to differ significantly. In contrast, the mRED Score for male embryos in Replicate 4 (0.94±0.19) was significantly lower compared with the mRED Score for embryos of the same sex in Replicate 2, but not Replicates 1 and 3.

The sex ratios of embryos originating from oocytes derived from the left and right ovaries after various periods of in vitro maturation is presented in timeline format in Figure 7.17. Additionally, timelines for mRED Scores for male and female embryos originating from the left and right ovaries is also presented in Figure 7.18.

**Discussion**

The overall sex ratio of the IVF embryos produced in Experiment 7.1 was 43.4% and was significantly lower than the expected ratio of 50%. This is in sharp contrast to previously published reports in which the sex ratio of IVP embryos was either significantly higher than parity (Lonergan et al., 1999) or did not differ from the expected sex ratio of 50% (Carvalho et al., 1996; Gutierrez-Adan et al, 2001; Agung et al., 2005). The mechanism underlying the low sex ratio distortion observed in this study remains unknown. This sex ratio accounts for all embryos produced and is not reflective of embryos that developed to an arbitrary end point (i.e., day 7) and therefore, presents a good indicator of the sex ratio of the embryos produced immediately following fertilization, as opposed to only viable embryos after an extended period of culture (i.e., blastocyst).

The duration of gamete interaction during in vitro fertilization in cattle has been implicated as influencing the embryo sex ratio in IVP systems (Kochhar et al., 2003), in which a co-incubation length of sperm and oocytes of 6 hours or less produced embryos with a significant male-bias. The co-incubation length in the present experiment was ~6
Figure 7.16. Mean (±SEM) mRED Scores by replicate of male and female embryos after all time periods of in vitro maturation. *ab* Bars with different letters are significantly different (P<0.05).
Figure 7.17. Timeline for sex ratios (% male) of embryos derived from oocytes originating from the left and right ovaries after in vitro maturation for different periods.

Points within ovaries with different letters are significantly different (P<0.05).

Points between ovaries with different letters are significantly different (P<0.05).
Figure 7.18. Timeline of mRED Scores for male and female embryos derived from oocytes originating from the left and right ovaries after in vitro maturation for different periods. a,b Points within sexes with different letters are significantly different (P<0.05). c,d Points between sexes with different letters are significantly different (P<0.05). e,f Points within ovaries with different letters are significantly different (P<0.05). g,h Points between ovaries with different letters are significantly different (P<0.05).
hours. Therefore, the results of Kochhar et al. (2003) cannot be confirmed with this experiment, as evidenced by the low sex ratio detected in the present study. Similarly, sperm pre-incubation length prior to insemination in cattle has been reported to affect the sex ratio of embryos produced in vitro (Lechniak et al., 2003).

Embryos with a female-biased sex ratio were produced following insemination with sperm pre-incubated for 24 hours, while insemination with sperm not subjected to a pre-incubation period produced embryos with a sex ratio that did not differ from parity. The spermatozoa used in the present experiment were not subjected to an extended incubation period prior to being co-incubated with the oocytes.

The overall sex ratio of the IVF embryos produced in Experiment 7.2 was 45.9% and was not significantly different from the expected ratio of 50%. These data confirm previous reports in which the sex ratio of bovine embryos produced in vitro did not differ from the expected sex ratio of 50% (Carvalho et al., 1996; Gutierrez-Adan et al. 2001; Agung et al., 2005).

Male embryos have been reported to develop faster than female embryos (Avery et al., 1989; Xu et al., 1992; Tominaga et al., 1996). The results from Experiments 7.1 and 7.2 did not demonstrate the ability of male embryos to develop faster than female embryos, as determined by mRED Scores (P=0.09 and 0.11, respectively). However, the absolute mRED Score values for male embryos were higher than female embryos in both experiments and additional observations may demonstrate potential differences in developmental rates of male and female embryos.

No difference was detected in the sex ratio of embryos from the left and right ovaries. In addition, the sex ratios from both ovaries did not differ significantly from the expected ratio of 50%. However, the failure to detect any difference may be the result of an insufficient number of embryos in both experiments.

In humans, the sex ratio of offspring has been described as “U-shaped” following insemination relative to ovulation (Guerrero, 1974; Harlap, 1979; James, 1994), with a male-bias produced from inseminations early and immediately prior to ovulation. Inseminations conducted between the early and immediately prior periods tend to produce offspring with a female-bias. Correspondingly, a similarly shaped pattern of sex ratios has been proposed in cattle (Denis, 1978).
If the differences observed are the result of varying maturational states of oocytes, then the sex ratio of embryos produced following sequential maturation periods should display a similar sex ratio pattern. The results of such a plot from the present study are shown in Figure 7.17. The sex ratios of embryos derived from the left and right ovaries failed to follow an inverted “U-shaped” pattern. In a similar manner, the mRED Scores of male and female embryos derived from the left and right ovaries are plotted against maturation duration in Figure 7.18. Again, no discernable pattern is evident between the categories of sex and ovary side.

The results of these experiments fail to provide insight into the relationship between the ovaries of origin and the sex ratio of the subsequently derived bovine embryos following IVF. In addition, the sexually dimorphic developmental inequalities previously reported in the scientific literature were not encountered in the present studies. Similarly, these data do not compare favorably with laterally asymmetric sex ratio distributions reported in the rabbit (YoungLai, 1981), mouse (Endo et al, 1987; Sakai and Endo, 1987) and Mongolian gerbil (Clark and Galef, 1990, 1995; Clark et al., 1994) and those detected previously in Experiments 3 and 5.
Animal production systems would benefit greatly, both genetically and financially, with the ability to effectively select the desired sex prior to conception. However, the only reliable sex selection technique, using the FACS approach, is hampered with high production costs and limited sorting rates. In an effort to overcome the shortcomings of flow-cytometrically sorted semen, investigations of alternative sex selection techniques are warranted. An asymmetric distribution of the sexes within the left and right uterine horns has been described in multiple laboratory species. However, no information is available regarding the lateral distribution of the sexes in cattle. A series of experiments were conducted to evaluate the sex ratio (% male) of calves gestated in the left and right uterine horns. In addition, the sex ratio of in vivo-derived and in vitro-derived embryos originating from the left and right ovaries of cattle was also evaluated.

In Experiment 1, the sex ratio of calves and fetuses gestated in the left and right uterine horns was investigated. The sex ratio of calves and fetuses gestated in the right uterine horn was significantly higher compared with the sex ratio of calves and fetuses gestated in the left uterine horn. In addition, the sex ratio of the left and right uterine horns was significantly different from parity. The results from this experiment compare favorably with those previously reported in the mouse (Endo et al., 1987) and Mongolian gerbil (Clark and Galef, 1991).

In Experiment 2, embryo transfer records were analyzed in an effort to determine if sex-specific selection pressures were applied to embryos in the uterine horn of transfer. The sex ratio of ET calves born following transfer to the left and right uterine horns was not significantly different. Similarly, the overall sex ratio of calves born in this experiment was not significantly different from parity. The lack of a distorted sex ratio resulting from either left horn or right horn embryo transfers at day 7 indicates that individual uterine horns do not apply sex-specific selection pressures that would increase the mortality of embryos of one sex.

To avoid the possibility of sex-specific embryonic loss described by Gosling (1998) in the nutria (Myocastor coypus), the sex ratios of embryos recovered from the
left and right uterine horns of superovulated beef cows were evaluated in Experiment 3. The proportion of male embryos collected from the right uterine horns was significantly greater than the proportion of male embryos from the left uterine horns. Clark et al. (1994) demonstrated that the ovary of origin was partially responsible for the distorted sex ratios observed in the uterine horns. Because transuterine migration is rare in cattle, the embryos recovered from each uterine horn provides insight to the ipsilateral ovary. The results of this experiment support the hypothesis of Clark et al. (1994) that the ovary of origin may influence the sex of the offspring.

In an effort to determine the role of interovarian communications in the sex selection process, the sex ratio of embryos recovered from unilaterally ovariectomized superovulated beef heifers was investigated in Experiment 4. The sex ratios of embryos recovered from left-ovary and right-ovary intact heifers was not significantly different. This lack of a sex ratio difference may be the result of a lack of sufficient observations. To circumvent the poor response from heifers found in this experiment, the use of animals with a known reproductive history should be used in future investigations.

In Experiment 5, the sex ratio of IVP embryos was evaluated. A review of the literature suggests that most IVP sytems produce embryos with a sex ratio that are either not different from parity or significantly higher than parity. The sex ratio of the IVP embryos originating from the left and right ovaries produced in this experiment was significantly lower than parity and was not different between ovaries of origin. In addition, the duration of in vitro oocyte maturation has been implicated as influencing the sex ratio of the subsequently derived embryos. The results of this experiment, however, indicate that length of in vitro oocyte maturation did not influence the sex ratio of IVF-derived bovine embryos.

The results of these experiments clearly demonstrate that a significantly greater proportion of males are gestated in the right uterine horn of cattle and a greater proportion of females in the left. Given the low incidence of transuterine migration and the absence of selection pressure on transferred embryos, there is a high probability that the altered sex ratios found in these studies are the result of the ovary of origin. Though the sex ratio of embryos recovered from the uterine horns was significantly different, the distortion was not as severe in superovulated donors compared with naturally mated
females. In addition, this lateral asymmetry was not detected in vitro. Perhaps, as conditions deviate from a natural breeding environment through the use of exogenous gonadotropins and in vitro culture conditions, the mechanisms responsible for the skewed sex ratios found in Experiment 3 become shrouded.
LITERATURE CITED


Clark, M.M. and B.G. Galef, Jr. 1990. Sexual segregation in the left and right horns of the gerbil uterus: "the male embryo is usually on the right, the female on the left" (Hippocrates). Dev. Psychobiol. 23:29-37.


James, W.H. 1996. The evidence that mammalian sex ratios at birth are partially controlled by maternal hormone levels at the time of conception. J. Theor. Biol. 130:397-404.


### APPENDIX: SUPPLEMENTAL MEDIA AND STOCK SOLUTIONS

#### A

**EMBRYO COLLECTION MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPBS</td>
<td>21300-025</td>
<td>Gibco</td>
<td>1,000 ml</td>
</tr>
<tr>
<td>BCS</td>
<td>SH30118</td>
<td>HyClone</td>
<td>10 ml</td>
</tr>
<tr>
<td>Pen-Strep Stock²</td>
<td>15140-122</td>
<td>Gibco</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

¹ Do not add BCS and antibiotic until day of use.
² Pen-Strep is purchased as a 100X stock solution from the manufacturer.

#### B

**EMBRYO HOLDING MEDIUM**

<table>
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<th>Company</th>
<th>Amount</th>
</tr>
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<td>DPBS</td>
<td>21300-025</td>
<td>Gibco</td>
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<tr>
<td>BCS</td>
<td>SH30118</td>
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<td>Pen-Strep Stock²</td>
<td>15140-122</td>
<td>Gibco</td>
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</table>

¹ Add antibiotic and BCS on day of use.
² Pen-Strep is purchased as a 100X stock solution from the manufacturer.

#### C

**1.4 M GLYCEROL EMBRYO FREEZE MEDIUM**

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<td>DPBS</td>
<td>21300-025</td>
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<td>8.9 ml</td>
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<td>Glycerol</td>
<td>G5516</td>
<td>Sigma</td>
<td>1.0 ml</td>
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<td>FBS</td>
<td>SH30088</td>
<td>HyClone</td>
<td>100 µl</td>
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</tbody>
</table>

¹ Prepare on day of use. Combine all ingredients in a 15-ml conical tube. Invert gently to dissolve.
1.0 M SUCROSE EMBRYO THAW MEDIUM¹

<table>
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<td>HyClone</td>
<td>250 µl</td>
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<tr>
<td>Sucrose</td>
<td>S1888</td>
<td>Sigma</td>
<td>8.55 g</td>
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<td>DPBS</td>
<td>21300-025</td>
<td>Gibco</td>
<td>25 ml</td>
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¹ Prepare on day of use. Combine FBS and sucrose in a 50-ml conical tube with ~20 ml DPBS. Invert gently to dissolve. Q.S. to 25 ml.

GEL LOADING BUFFER¹

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<tr>
<th>Component</th>
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<th>Amount</th>
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<tbody>
<tr>
<td>ddH₂O</td>
<td>W3500</td>
<td>Sigma</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sucrose</td>
<td>S1888</td>
<td>Sigma</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Cresol Red Stock</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

¹ Add sucrose to ddH₂O in 50-ml conical tube. Shake to dissolve. Add Cresol Red stock (Appendix U). Invert tube gently to mix. Store at 4°C.

3% AGAROSE ELECTROPHORESIS GEL¹

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50X TAE Stock²</td>
<td>-</td>
<td>-</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>W3500</td>
<td>Sigma</td>
<td>98.0 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>A9918</td>
<td>Sigma</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>46067</td>
<td>Sigma</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

¹ Combine TAE stock (Appendix W), ddH₂O and agarose in 250-ml flask. Heat to dissolve, stirring occasionally. Once dissolved, add EtBr and swirl to mix. Cool to 70°C before pouring into mold.

² 10 ml of 10X TBE Stock (Appendix X) may be substituted. If used, reduce amount of ddH₂O to 90 ml.
### OVARY TRANSPORT MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>4.3092 g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>W-3500</td>
<td>Sigma</td>
<td>500 ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

1 Sterilize in autoclave prior to use. Add antibiotic after sterilization and not until day of use.

### OOCYTE HOLDING MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL-HEPES</td>
<td>04-616F</td>
<td>Cambrex</td>
<td>45 ml</td>
</tr>
<tr>
<td>FBS</td>
<td>SH30088</td>
<td>HyClone</td>
<td>5 ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

1 Prepare on day of use. Place in a 37°C water bath at least 20 minutes prior to use.
**OOCYTE MATURATION MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>11150-059</td>
<td>Gibco</td>
<td>8.835 ml</td>
</tr>
<tr>
<td>FSH Stock</td>
<td>-</td>
<td>-</td>
<td>0.020 ml</td>
</tr>
<tr>
<td>LH Stock</td>
<td>-</td>
<td>-</td>
<td>0.125 ml</td>
</tr>
<tr>
<td>E₂ Stock</td>
<td>-</td>
<td>-</td>
<td>0.010 ml</td>
</tr>
<tr>
<td>FBS SH30088</td>
<td>HyClone</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>0.010 ml</td>
</tr>
<tr>
<td>Mineral Oil</td>
<td>M-5310</td>
<td>Sigma</td>
<td>-</td>
</tr>
</tbody>
</table>

**Preparation Instructions:**

1. Combine 8.835 ml TCM-199, 0.020 ml FSH Stock (Appendix Y), 0.125 ml LH Stock (Appendix Z), 1.0 ml FBS and 0.010 ml Gentamicin in 15-ml conical tube. Sterile filter (0.2 µm).
2. Add 0.010 ml E₂ Stock (Appendix AA).
3. Prepare 50-µl maturation droplets under mineral oil and allow to equilibrate in incubator at 38°C and 5% CO₂ for a minimum of 3 hours prior to use.
4. Remaining medium can be used to wash COCs prior to placement in maturation droplets.

**BO-A STOCK SOLUTION¹**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>4.3092 g</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>0.1974 g</td>
</tr>
<tr>
<td>CaCl₂ • 2H₂O</td>
<td>C-7902</td>
<td>Sigma</td>
<td>0.2171 g</td>
</tr>
<tr>
<td>NaH₂PO₄ • H₂O</td>
<td>S-9638</td>
<td>Sigma</td>
<td>0.0743 g</td>
</tr>
<tr>
<td>MgCl₂ • 6H₂O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>0.0697 g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>W-3500</td>
<td>Sigma</td>
<td>500 ml</td>
</tr>
<tr>
<td>Phenol Red  0.5%</td>
<td>P-0290</td>
<td>Sigma</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

¹ Solution may be stored at 4°C for up to 3 months.
### BO-B STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO(_3)</td>
<td>S-5886</td>
<td>Sigma</td>
<td>2.5873 g</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>W-3500</td>
<td>Sigma</td>
<td>200 ml</td>
</tr>
<tr>
<td>Phenol Red 0.5%</td>
<td>P-0290</td>
<td>Sigma</td>
<td>0.04 ml</td>
</tr>
</tbody>
</table>

\(^1\) Prepare and store in a 250-ml bottle. Inject CO\(_2\) gas into solution until color change occurs. May be stored at 4\(^\circ\)C for up to 3 months.

### CR1aa STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>0.6703 g</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>0.0231 g</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>S-8875</td>
<td>Sigma</td>
<td>0.2201 g</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>P-4562</td>
<td>Sigma</td>
<td>0.0044 g</td>
</tr>
<tr>
<td>L(+)Lactic Acid</td>
<td>L-4388</td>
<td>Sigma</td>
<td>0.0546 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>G-8790</td>
<td>Sigma</td>
<td>0.0039 g</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>A-7469</td>
<td>Sigma</td>
<td>0.0045 g</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>W-3500</td>
<td>Sigma</td>
<td>100 ml</td>
</tr>
<tr>
<td>Phenol Red 0.5%</td>
<td>P-0290</td>
<td>Sigma</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

\(^1\) Solution may be stored at 4\(^\circ\)C for up to 3 months.
### Fertilization Medium (BO-AB)<sup>1</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-A Stock</td>
<td>-</td>
<td>-</td>
<td>38 ml</td>
</tr>
<tr>
<td>BO-B Stock</td>
<td>-</td>
<td>-</td>
<td>12 ml</td>
</tr>
<tr>
<td>Heparin Sodium</td>
<td>53312S</td>
<td>Elkins-Sinn</td>
<td>18 µl</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>P-4562</td>
<td>Sigma</td>
<td>0.0069 g</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

<sup>1</sup> Prepare on day of use. Combine BO-A Stock (Appendix J) and BO-B Stock (Appendix K) in 50-ml conical tube. Add remaining ingredients and invert gently to dissolve.

### BO-Caffeine Solution<sup>1</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-AB</td>
<td>-</td>
<td>-</td>
<td>25 ml</td>
</tr>
<tr>
<td>Caffeine Sodium</td>
<td>C-4144</td>
<td>Sigma</td>
<td>0.0243 g</td>
</tr>
</tbody>
</table>

<sup>1</sup> Prepare on day of use. Add BO-AB (Appendix M) and Caffeine Sodium to a 50-ml conical tube. Invert gently to dissolve. Sterile filter and place in 37°C water bath until needed.

### BSA-BO (0.6%) Solution<sup>1</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-AB</td>
<td>-</td>
<td>-</td>
<td>5 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511</td>
<td>Sigma</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

<sup>1</sup> Prepare on day of use. Add BO-AB (Appendix M) and BSA to a 15-ml conical tube. Invert gently to dissolve. Sterile filter and place in a 38°C incubator at 5% CO₂ to equilibrate.
BSA-BO (0.3%) SOLUTION

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-AB</td>
<td>-</td>
<td>-</td>
<td>10 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511</td>
<td>Sigma</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

1 Prepare on day of use. Add BO-AB (Appendix M) and BSA to a 15-ml conical tube. Invert gently to dissolve. Sterile filter and place in a 38°C incubator at 5% CO₂ to equilibrate.

OOOCYTE DENUDING MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>11150-059</td>
<td>Gibco</td>
<td>10 ml</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>H-3506</td>
<td>Sigma</td>
<td>0.012 g</td>
</tr>
</tbody>
</table>

1 Prepare on day of use in a 15-ml conical tube, sterile filter and place in a 38°C incubator at 5% CO₂ to equilibrate.

OOOCYTE WASH MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>11150-059</td>
<td>Gibco</td>
<td>10 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511</td>
<td>Sigma</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

1 Prepare on day of use in a 15-ml conical tube, sterile filter and place in a 38°C incubator at 5% CO₂ to equilibrate.
### IVC MEDIUM (Day 0 to 3)\(^1,2\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td></td>
<td></td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BMEaa</td>
<td>B-6766</td>
<td>Sigma</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEMaa</td>
<td>11140-050</td>
<td>Gibco</td>
<td>100 µl</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>G-5763</td>
<td>Sigma</td>
<td>0.0015 g</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511</td>
<td>Sigma</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

\(^1\) Rosenkrans and First (1994).

\(^2\) Prepare culture medium on day of use. Add CR1aa Stock (Appendix L) and remaining ingredients to a 15-ml conical tube. Invert gently to dissolve. Sterile filter and equilibrate in a 38°C, 5% CO\(_2\) incubator.

### IVC MEDIUM (Day 3 to 7)\(^1,2\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td></td>
<td></td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BMEaa</td>
<td>B-6766</td>
<td>Sigma</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEMaa</td>
<td>11140-050</td>
<td>Gibco</td>
<td>100 µl</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>15750-060</td>
<td>Gibco</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>G-5763</td>
<td>Sigma</td>
<td>0.0015 g</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511</td>
<td>Sigma</td>
<td>0.03 g</td>
</tr>
<tr>
<td>FBS</td>
<td>SH30088</td>
<td>HyClone</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

\(^1\) Rosenkrans and First (1994).

\(^2\) Prepare culture medium on day of use. Add CR1aa Stock (Appendix L) and remaining ingredients to a 15-ml conical tube. Invert gently to dissolve. Sterile filter and equilibrate in a 38°C, 5% CO\(_2\) incubator.
### 1% CRESOL RED STOCK\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH(_2)O</td>
<td>W3500</td>
<td>Sigma</td>
<td>50 ml</td>
</tr>
<tr>
<td>Cresol Red</td>
<td>114472</td>
<td>Sigma</td>
<td>500 mg</td>
</tr>
</tbody>
</table>

\(^1\) Combine in 50-ml conical tube. Shake to dissolve. Store at room temperature.

### 0.5 M EDTA STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>E6758</td>
<td>Sigma</td>
<td>186.1 g</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>W3500</td>
<td>Sigma</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

\(^1\) Combine EDTA and ~800 ml ddH\(_2\)O. Stir vigorously to dissolve. Adjust pH to 8.0. Q.S. to 1,000 ml. Autoclave and store at room temperature.

### 50X TAE BUFFER STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Base</td>
<td>T1503</td>
<td>Sigma</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>A6283</td>
<td>Sigma</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>0.5 M EDTA Stock</td>
<td>-</td>
<td>-</td>
<td>100 ml</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>W3500</td>
<td>Sigma</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

\(^1\) Add EDTA Stock (Appendix V), TRIS Base, Acetic acid to ~800 ml ddH\(_2\)O. Invert gently to dissolve. Adjust pH to 8.5. Q.S. to 1,000 ml. Store at room temperature.
### 10X TBE BUFFER STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Base</td>
<td>T1503</td>
<td>Sigma</td>
<td>108 g</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>B6768</td>
<td>Sigma</td>
<td>55 g</td>
</tr>
<tr>
<td>0.5 M EDTA Stock</td>
<td>-</td>
<td>-</td>
<td>40 ml</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>W3500</td>
<td>Sigma</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

\(^1\) Add EDTA Stock (Appendix V), TRIS Base, Boric acid to ~800 ml ddH\(_2\)O. Invert gently to dissolve. Adjust pH to 8.3. Q.S. to 1,000 ml. Store at room temperature.

### FSH STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFSH</td>
<td>F-2293</td>
<td>Sigma</td>
<td>490 IU</td>
</tr>
<tr>
<td>TCM-199</td>
<td>11150-059</td>
<td>Gibco</td>
<td>14 ml</td>
</tr>
</tbody>
</table>

\(^1\) One vial contains 490 IU (~35 mg). Reconstitution with 14 ml of TCM-199 yields a 2.5 mg/ml stock (35 IU/ml). Store at -80°C in 50-\(\mu\)l aliquots.

### LH STOCK SOLUTION\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLH</td>
<td>L-9773</td>
<td>Sigma</td>
<td>30,000 IU</td>
</tr>
<tr>
<td>TCM-199</td>
<td>11150-059</td>
<td>Gibco</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

\(^1\) One vial contains 30,000 IU (~8 mg). Reconstitution with 10 ml of TCM-199 yields a 0.8 mg/ml stock (3000 IU/ml). Store in 200-\(\mu\)l aliquots -80°C.
## $E_2$ STOCK SOLUTION

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>E-2758</td>
<td>Sigma</td>
<td>0.010 g</td>
</tr>
<tr>
<td>Ethanol</td>
<td>E-7148</td>
<td>Sigma</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

* Store at -80°C in 50-μl aliquots. Discard after 4 weeks.
VITA

Darin Alan Hylan was the first child born to Roger Alan and Elizabeth Louise Sistrunk Hylan in Homer, Louisiana, on March 12, 1970. Due to the employment obligations of his father, Darin received his elementary and secondary education from various schools located in Louisiana.

After graduating from Winnfield Senior High School in May 1988, he attended Louisiana Tech University where he earned his Bachelor of Science in animal science in August, 1995. While pursuing his undergraduate degree, he worked as an embryo transfer technician at Advanced Applied Genetics, a commercial embryo transfer company, based in Franklin, Louisiana.

In August of 1996, he entered graduate school at Louisiana State University under the direction of Dr. Robert A. Godke. Darin was awarded a Master of Science in animal science in the Fall of 2000. In August, 1998, Darin became a Research Associate for the LSU Agricultural Center at Dean Lee Research Station located in Alexandria, Louisiana.

In March of 2003, Darin became the Andrologist at Woman’s Center for Fertility and Reproductive Medicine at Woman’s Hospital in Baton Rouge, Louisiana. He entered Graduate School in June of 2001 in pursuit of a Doctor of Philosophy degree in reproductive physiology under the direction of Dr. Robert A Godke, Boyd Professor, and is now a candidate for the Doctor of Philosophy degree.