

12-2009

Discovering Metabolic Networks of Bovine Fertilization

Erin Lynn Young
Utah State University

Follow this and additional works at: <http://digitalcommons.usu.edu/honors>

 Part of the [Plant Sciences Commons](#)

Recommended Citation

Young, Erin Lynn, "Discovering Metabolic Networks of Bovine Fertilization" (2009). *Undergraduate Honors Theses*. Paper 29.

This Thesis is brought to you for free and open access by the Honors Program at DigitalCommons@USU. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of DigitalCommons@USU. For more information, please contact becky.thoms@usu.edu.



**DISCOVERING METABOLIC NETWORKS OF BOVINE
FERTILIZATION**

by

Erin Lynn Young

**Thesis submitted in partial fulfillment
of the requirements for the degree**

of

**HONORS IN UNIVERSITY STUDIES
WITH DEPARTMENTAL HONORS**

in

**Bioveterinary Science
in the Department of Animal, Dairy & Vet Sciences**

Approved:

Thesis/Project Advisor
Dr. Lee Rickords

Departmental Honors Advisor
Dr. Lee Rickords

Director of Honors Program
Dr. Christie Fox

**UTAH STATE UNIVERSITY
Logan, UT**

Fall 2009

Abstract

At the time of fertilization, a dramatic change occurs in the oocyte that transforms this cell from a metaphase arrested state into a metabolically active and dynamic state. The view of the flow of biological processes within organisms has recently shifted from that of a linear path to a more complex network. Biological processes are no longer thought of in the simple terms of DNA to RNA, RNA to proteins, and proteins to final activity. It is now known that many biological processes involve interconnected networks and feedback loops in which DNA, RNA, proteins, and metabolites perform specific roles. We hypothesized that there are key metabolites and metabolic pathways yet to be determined that are involved in normal mammalian fertilization and embryonic development.

The key objective of this research project was to study the metabolic profiles of unfertilized oocytes and fertilized zygotes to find the differences in the concentration and flux of key metabolites. Examining these differences provides evidence for which metabolic pathways are important during fertilization.

Concentration differences of metabolites were assessed within unfertilized bovine oocytes and *in vitro* fertilized zygotes. Metabolites were identified using gas chromatography-mass spectrometry (GC/MS). Differences in metabolite pools pre- and post-fertilization may be used as intracellular biomarkers of normal embryonic development. It was found that D-glucose, arabinose and fumaric acid were present in lower concentrations in the zygote than the unfertilized oocytes.

Acknowledgments

We wish to thank Dr. Lee Rickords, Benson Morrill, Davin Larsen, Kim Elwood and Marcy Labrum, Utah State University, Logan, UT, for assistance with GC/MS analysis of samples, Dr. Ken White and Ben Sessions for oocyte and zygote collection and URCO (Undergraduate Research & Creative Opportunities) for funding.

Table of Contents

- 5. List of Tables and Figures
- 6. Text
 - 6. Introduction
 - 11. Materials and Methods
 - 12. Results
 - 13. Conclusion
- 15. Sources Cited
- 17. Author's Biography

List of Tables and Figures:

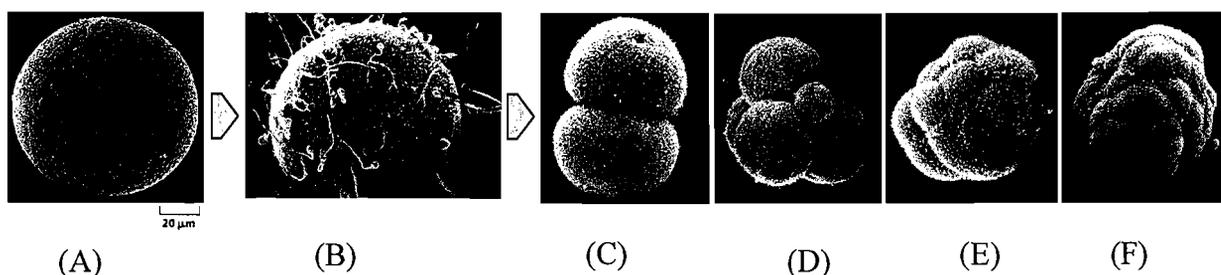
6. Figure 1. Changes Induced on an Oocyte Upon Fertilization
10. Figure 2. A Diagram of a Gas Chromatography Mass Spectrometry Machine
12. Figure 3. The Resulting Chromatograms of the Metabolites of the Collected Oocytes and Zygotes
13. Figure 4. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram.
13. Table 1. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram.
14. Figure 5. The Chemical Structures of the Compounds Identified as Significant
17. Image 1. A Photograph of Erin Young, the Author

Thesis Text

Introduction

Fertilization is the beginning of embryogenesis. An oocyte and sperm fuse to form a zygote, which divides repeatedly in order to produce a new individual/organism (Alberts *et al.*, 2008b). At the time of fertilization, a dramatic change occurs in the oocyte that advances this cell from a metaphase II arrested state into a metabolically active and dynamic state (see **Figure 1**). It is presumed that several metabolic pathways are initiated upon fertilization. Relatively little information is available about specific metabolites and their networks during mammalian fertilization.

Figure 1. Changes Induced on an Oocyte Upon Fertilization. Upon fertilization, an oocyte moves out of a metabolically inactive state and begins to grow and divide, thus becoming a new organism. (A) an oocyte, (B) an oocyte surrounded by sperm depicting fertilization, (C - F) a depiction of the division that takes place after fertilization, the initial stages of embryogenesis (adapted from Alberts *et al.*, 2008a; Alberts *et al.*, 2008b, copyright Garland Science 2008).



An oocyte is the female germ cell. The oocyte acts as a giant storehouse of materials, thus making it possible to divide into many smaller cells quickly upon activation (Alberts *et al.*, 2008b). Upon oocyte maturation, an oocyte is arrested in meiosis metaphase II (Alberts *et al.*, 2008b, Ferreira *et al.*, 2009). The oocyte stores transcripts and proteins in the cell's cytoplasm.

These stored compounds are of great importance for ensuring a normal, healthy progression as embryogenesis begins. Oocytes also store cortical granules, which are derived from the Golgi complex in the cell. They are composed of proteins, structural molecules and enzymes. When the oocyte reaches metaphase II in the meiosis cycle, these granules are dispersed throughout the inner surface of the cell, close to the plasma membrane (Ferreiraa *et al.*, 2009).

Fertilization is the process in which an oocyte, the female gamete, and a sperm, the male gamete, fuse. After the fusion of the sperm and oocyte, all of the sperm contents are absorbed by the oocyte. This activates the oocyte, causing the cortical granules stored inside to release their contents (Alberts *et al.*, 2008a). Upon fertilization, these granules undergo exocytosis, thus changing the plasma membrane of an oocyte which prevents polyspermy (Ferreiraa *et al.*, 2009). When the sperm fuses with the plasma membrane of the oocyte, the concentration of inositol 1,4,5, trisphosphate (IP3) increases, which releases calcium that has been stored from the endoplasmic reticulum of the oocyte. The increase of cytosolic calcium concentration causes additional calcium channels to open, which allows more calcium to enter the cytosol. The oocyte resumes meiosis and produces a second polar body (Alberts *et al.*, 2008a).

Upon sperm-oocyte fusion, the oocyte is renamed a zygote. A zygote still consists of only a single cell and has not undergone implantation. At fertilization, the sperm contributes a haploid nucleus, called a pronuclei. Fertilization is not complete until the pronuclei of the zygote and sperm fuse. For mammals, the two pronuclei do not fuse until the zygote undergoes its first mitotic division, forming a diploid genome of a new organism (Alberts *et al.*, 2008a). The zygote is then fully active in its developmental program (Alberts *et al.*, 2008b).

Once the oocyte has been fertilized, the cell divides, producing many cells from the original. These new cells specialize and differentiate to perform specific biological functions.

They interact with their neighboring cells and migrate to specific regions necessary for normal development. This all begins to happen at fertilization, and is common to almost all animals (Alberts *et al.*, 2008a).

Metabolic pathways act as the sources of building blocks -- sugars, amino acids, and lipids-- that form cellular structures and macromolecules for regulatory function for the cells in all organisms. They allow the assimilation of simple and modified monosaccharides, amino acids, nucleotides and modified lipids to form complex molecules such as polysaccharides, lipids, and proteins.

The view of the flow of biological processes within organisms has recently shifted from that of a linear path to a more complex network. Biological processes are no longer thought of in the simple terms of DNA to RNA, RNA to proteins, and proteins to final activity. It is now known that many biological processes involve interconnected networks and feedback loops in which DNA, RNA, proteins, and metabolites perform specific roles (Barabási, 2004). While genomics, the study of an organism's complete set of genes, and proteomics, the study of an organism's complete set of proteins, have proven to be helpful in understanding the functions of many genes and proteins in various biological systems, it has also been shown that differences in gene transcription and protein levels do not necessarily lead to changes in phenotype (Sumner *et al.*, 2003; Gygi *et al.*, 1999). The field of metabolomics offers end-point analysis of metabolites at a specific time within the organism and therefore provides a unique approach for comprehending how genes and environment affect the cellular phenotype.

Although we have reached a point in the study of mammalian reproduction where we are able to understand and manipulate many reproductive processes, the low efficiencies of success with manipulating these processes, such as somatic cell nuclear transfer (mammalian cloning)

(Beyhan et al., 2007), provide compelling evidence that our understanding of these processes is still lacking. Therefore, we hypothesized that small molecule metabolites and their pathway networks play a critical role in normal mammalian fertilization. To test this hypothesis, we investigated the differences in metabolites present in a mature oocyte and those present in a zygote. The data obtained from these experiments will provide important information regarding metabolic pathways that are vital to mammalian fertilization and early development. This would aid in improving media for other embryonic or stem cell studies, which could be used for various applications in both research and human health.

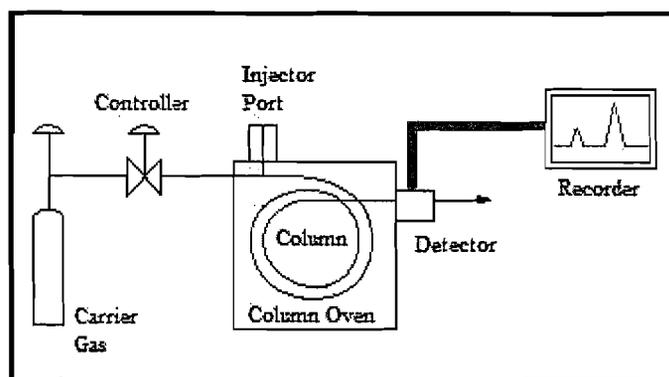
The bovine model was used to represent mammalian systems in this research project. One of the revelations of the past few decades has been that much of the basic machinery of development is essentially the same across species. Homologous proteins have been found to function interchangeably between very different species (Alberts *et al.* 2008b). The bovine model has been utilized for over twenty years and has achieved high *in vitro* developmental rates and good success to term (Reed *et al.*, 1996; Aston *et al.*, 2006a; Aston *et al.*, 2006b). Therefore, the bovine model is a good choice to study metabolites and cofactors associated with mammalian fertilization and early embryonic development.

While there is sufficient knowledge available on the involvement of small molecule substrates, relatively little is known about the exact role of these molecules in mammalian embryonic development (Sinclair *et al.*, 2003). We hypothesized that small molecule metabolites and their pathway networks were involved in normal fertilization and preimplantation embryo development. We tested our hypothesis using the bovine *in vitro* fertilization and culture model to determine the molecular events associated with mammalian development.

Recently, the development of high throughput technologies that allow large scale

profiling of physiology have facilitated rapid advances in understanding functions of cellular systems on a much broader scale. Gas chromatography has the potential to have high resolution, high efficiency, reproducibility, and small sample size (Issaq *et al.*, 2009). Gas chromatography coupled with mass spectrometry becomes a valuable and reliable analytical technique for separation, detection, and identification. GC/MS, however, is limited to small compounds that are thermally stable, volatile, or can be made volatile through derivatization (Issaq *et al.*, 2009). A sample destined for GC/MS is first derivatized, and then injected into a GC/MS machine (see **Figure 2**). The volatilized sample is carried by a carrier gas, normally helium, through a heated column where the compounds are separated by mass to charge ratio. A detector at the end of the column records the mass and time that each compound took to travel through the column. The readout of this process is a chromatogram (McLafferty, 1980).

Figure 2. A Diagram of a Gas Chromatography Mass Spectrometry Machine. A sample is injected into the machine, carried by a gas, normally helium, through a heated column. A detector at the end records the time and mass of each sample that exits the machine (adapted from weather.nmsu.edu)



Different types of cells from humans and other animals have been studied using this technique *in vivo*; examples include vascular smooth muscle cells, adipocytes, T-lymphocytes,

hippocampal neurons, hepatocytes, endothelial cells, mammary epithelial cells, the cerebral cortex, spermatocytes, pituitary tumors, chronic lymphocytic leukemia cells and others (Issaq *et al.*, 2009). Analysis by GC/MS is sensitive, reproducible and quantitative, and label incorporation and die-away approaches are simple to use and interpret.

Materials and Methods

Fertilized zygotes and unfertilized oocytes were provided by the laboratory of Dr. Kenneth L. White (Utah State University). There were 300 oocytes and 250 zygotes in each representative sample. Samples were frozen at -80°C in PB1 media until metabolite extraction was performed.

Ice cold 100% methanol was added to thawed cell samples to create a 50% methanol solution, after which cellular metabolites were extracted using an equivalent volume of chloroform.

Aqueous and organic layers were then separated and dried. Dry extracts were then derivatized using methoxyamine hydrochloride in pyridine, and incubated at 42° C for 90 minutes. Samples were silylated in N-Methyl-N-TMS-Trifluoroacetamide (MSTFA) at 42° C for 60 minutes. Because the low cell number of these oocyte samples did not give the sensitivity we expected, samples were concentrated by re-drying in a speedvac overnight and dissolved in 40 ul MSTFA and 60 ul pyridine.

After derivatization, samples were analyzed by GC/MS (GCT Premier, Waters) at the Center for Integrated BioSystems (CIB, Utah State University). 1 µl aliquots were injected into a DB5-MS capillary column (30 m x 250 µm i.d. x 0.25 µm film thickness). The initial GC oven

temperature was 70°C and 5 min after injection the temperature was increased 5°C/min to 310°C and held for 5 minutes at 310°C. Helium was used as a carrier gas and pressure was programmed such that the helium flow was kept constant at a flow rate of 1 ml/min. Detection was achieved using MS detection in electron impact mode and full scan monitoring mode (m/z 50-800). The temperature of the ion source was set at 250°C and to 200°C for the mass analyzer.

Results

A chromatogram was obtained by running samples through GC/MS (see **Figure 3**). This chromatogram was analyzed for differences in the concentrations of compounds between the oocytes and zygotes. The peaks were integrated to determine relative amounts (see **Table 1** and **Figure 4**).

Figure 3. The Resulting Chromatograms of the Metabolites of the Collected Oocytes and Zygotes. Chromatograms of Oocytes (top) and Zygotes (bottom). TIC – Total Ion current.

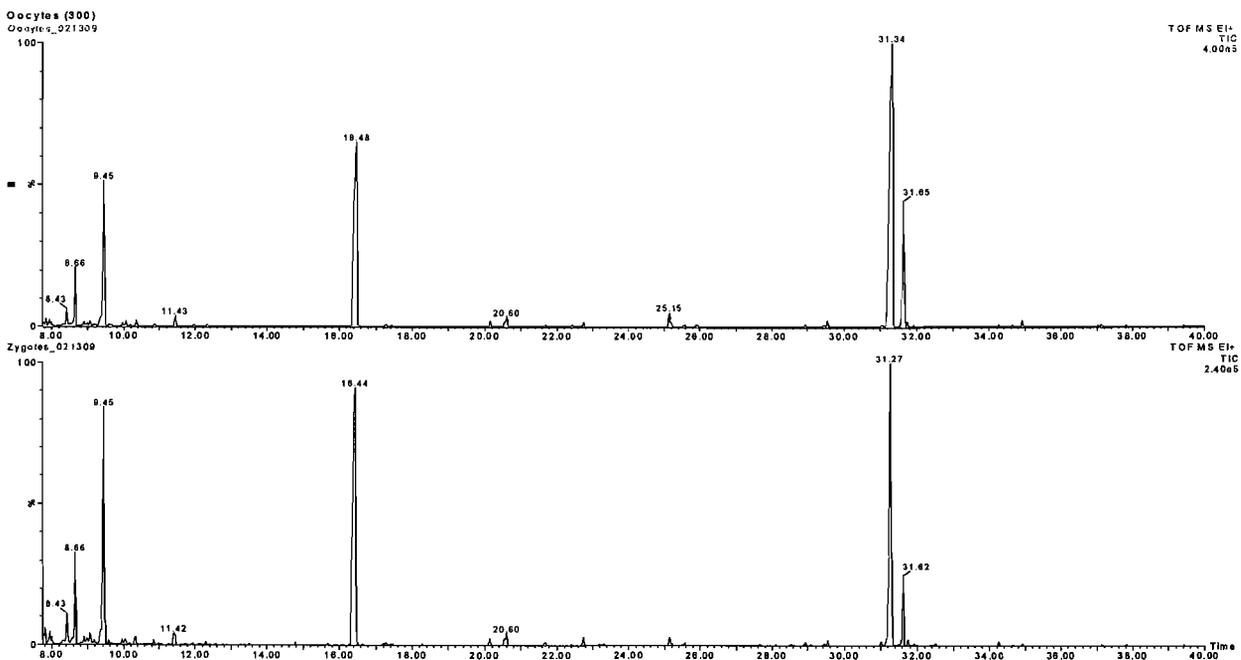
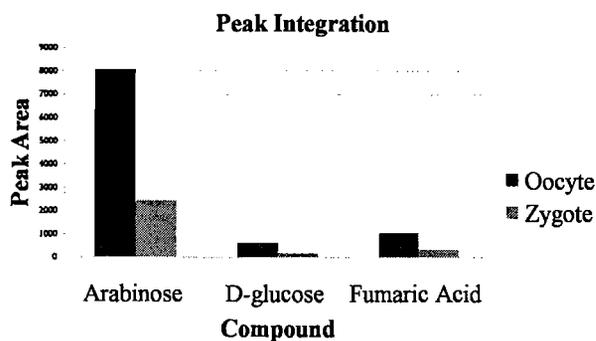


Figure 4. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram.

Comparisons of peak areas of bovine oocytes and zygotes of the compounds of interest.



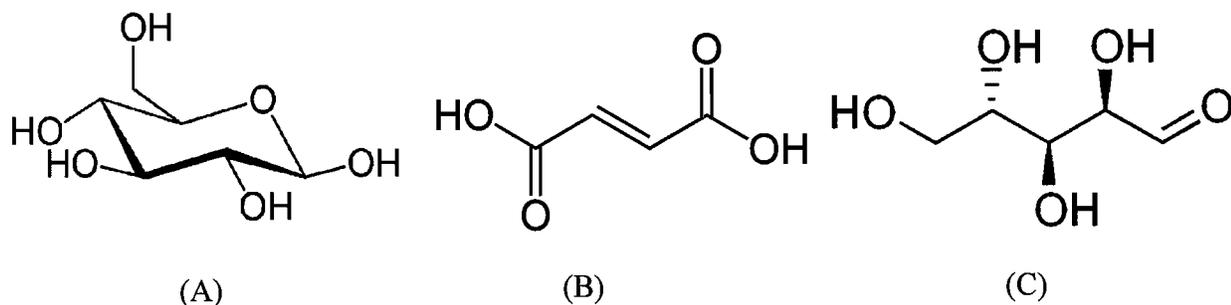
Compound	Oocyte Integration	Zygote Integration
Arabinose	8068	2450
D-glucose	639	161
Fumaric Acid	1055	315

Table 1. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram. A list of compounds found in the chromatogram with their corresponding peak integration values. A peak integration value is a measurement of peak area, which is considered proportional to the amount of compound present in each sample.

Conclusion

In an oocyte, important compounds are pooled and stored for the future developing embryo. Upon fertilization, the fusion of a sperm and oocyte causes the oocyte to become active and move out of Metaphase II. The zygote begins to use its stored compounds as embryogenesis begins. This is evidenced by a reduction in D-glucose, Arabinose, and Fumaric Acid (see **Figure 5**). Glucose is a major source of energy for a growing mammalian embryo.

Figure 5. The Chemical Structures of the Compounds Identified as Significant. (A) D-glucose, (B) Fumaric Acid and (C) Arabinose are involved in energy production. Arabinose is also involved in DNA synthesis.



D-glucose undergoes glycolysis and is used in the TCA cycle, which generates ATP, an energy source for the cell. Fumaric acid can be changed into fumarate, which is part of the TCA cycle and can also be used for energy (Kenealy *et al.* 1986). Arabinose can be isomerized by the Arabinose isomerase enzyme into D-ribose which is utilized in the pentose phosphate pathway to generate glucose and energy or used to synthesize nucleic acids, both of which would be needed in a rapidly growing and dividing cell population (Tozzi *et al.* 2006). When an oocyte is fertilized it is more active, and uses its stores of important compounds to grow and develop.

Biological samples are complex, and contain hundreds of metabolites which cannot be adequately separated using a single GC/MS experiment to detect all the metabolites (Issaq *et al.*, 2009). Although only one set of samples were run, and this study is not conclusive, we were able to identify three compounds that differed in concentration between the oocyte and zygote. In this study of the difference in metabolite concentrations of an oocyte pre- and post-fertilization, we saw evidence of significant metabolic activity of three specific compounds, arabinose, D-glucose and fumaric acid in the fertilized zygotes.

Sources Cited:

- Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. (2008a). "Sexual Reproduction: Meiosis, Germ Cells, and Fertilization." *Molecular Biology of the Cell, Fifth Edition*: 1269 - 1304.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. (2008b). "Development of Multicellular Organisms." *Molecular Biology of the Cell, Fifth Edition*: 1305 - 1416.
- Aston KI, Li GP, Hicks BA, Sessions BR, Pate BJ, Hammon D, Bunch TD and White KL. (2006a). "Effect of the time interval between fusion and activation on nuclear state and development in vitro and in vivo of bovine somatic cell nuclear transfer embryos." *Reproduction* **131**(1): 45 - 51.
- Aston KI, Li GP, Hicks BA, Sessions BR, Pate BJ, Hammon DS, Bunch TD and White KL. (2006b). "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." *Anim Reprod Sci* **95**(3-4): 234 - 43.
- Barabási A, Oltvai Z.(2004). "Network biology: understanding the cell's functional organization." *Nat Rev Genet* **5**(2): 101 - 13
- Beyhan Z, Ross PJ, Iager AE, Kocabas AM, Cunniff K, Rosa GJ, and Cibelli JB. (2007). Transcriptional reprogramming of somatic cell nuclei during preimplantation development of cloned bovine embryos. *Dev. Biol.* **305**(2): 637-49.
- Busch R, Kim Y, Neese R, Schade-Serin V, Collins M, Awada M, Gardner J, Beysen C, Marino M, Misell L *et al.* (2006). "Measurement of protein turnover rates by heavy water labeling of nonessential amino acids." *Biochim Biophys Acta* **1760**(5): 730 - 44.
- Busch R, Neese R, Awada M, Hayes G, Hellerstein M. (2007). "Measurement of cell proliferation by heavy water labeling." *Nat Protoc* **2**(12):3045 - 57.
- Campbell, KD, Reed WA and White KL. (2000). "Ability of integrins to mediate fertilization, intracellular calcium release, and parthenogenetic development in bovine oocytes." *Biol Reprod* **62**(6): 1702-9.
- Ferreira EM, Virequea AA, Adonab PR, Meirellesb FV, Ferriania RA and Navarro PAAS. (2009). "Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence." *Theriogenology*. **71**(5): 836-48.
- Gygi SP, Rochon Y, Franza BR, and Aebersold R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**: 1720 – 30.
- Issaq HJ, Van QN, Waybright TJ, Muschik GM and Veenstra TD. (2009) "Analytical and statistical approaches to metabolomics research." *Journal of Separation Science* **32**(13): 2183 - 99
- Kenealy W, Zaady E, Du Preez JC, Stieglitz B, and Goldberg I. (1986). "Biochemical Aspects of Fumaric Acid accumulation by *Rhizopus arrhizus*." *Applied and Environmental Microbiology* **52**: 128-133.
- McLafferty FW. (1980). "Introduction." *Interpretation of Mass Spectra*, Third Edition: 1-14
- Neese RA, Misell LM, Turner S, Chu A, Kim J, Cesar D, Hoh R, Antelo F, Strawford A, McCune JM. (2002) "Measurement in vivo of proliferation rates of slow turnover

cells by (H₂O)-H-2 labeling of the deoxyribose moiety of DNA." *Proceedings of the National Academy of Sciences of the United States of America* **99**(24): 15345 - 50.

- Reed, W A, Suh TK, Bunch TD and White KL. (1996). "Culture of in vitro fertilized bovine embryos with bovine oviductal epithelial cells, buffalo rat liver (brl) cells, or brl-cell-conditioned medium." *Theriogenology* **45**(2): 439 - 49.
- Sessions, BR, Aston KI, Davis AP, Pate BJ and White KL. (2006). "Effects of amino acid substitutions in and around the arginine-glycine-aspartic acid (rgd) sequence on fertilization and parthenogenetic development in mature bovine oocytes." *Mol Reprod Dev* **73**(5): 651 - 57.
- Sinclair, KD, Rooke JA and Mcevoy TG. (2003). "Regulation of nutrient uptake and metabolism in pre-elongation ruminant embryos." *Reprod Suppl* **61**: 371-85.
- Sumner LW, Mendes P, and Dixon RA. (2003). Plant metabolomics: largescale phytochemistry in the functional genomics era. *Phytochemistry* **62**: 817–36.
- Tozzi MG, Camici M, Mascia L, Sgarrella F and Ipata PL. (2006). "Pentose phosphates in nucleoside interconversion and catabolism." *FEBSJ* **273**(6):1089-101.

Biography:

Erin Young came to Utah State on a Presidential Scholarship for the opportunity to live away from home and develop her talents. She was introduced to undergraduate research her sophomore year. Erin found that she enjoyed the laboratory setting and her aspirations became more research oriented. She has spent time as an undergraduate researcher in both Dr. Quinton Winger's and Dr. Lee Rickord's laboratories. Erin has presented her research at the Utah Conference of Undergraduate Research and has been given the Undergraduate Research Scholar Transcript Designation. In addition to her experience at Utah State University, she was able to participate in the Integrated Cancer Biology Program's 2009 Summer Program, where she moved to Stanford University Campus to study acute myelogenous leukemia. Erin graduated with a Major in Bioveterinary Science with an emphasis in Biotechnology and minors in Biology, Chemistry and Mathematics. Erin grew up in Bountiful, Utah and graduated from Bountiful High School in 2004. Outside of class, Erin has a love for travel, theater and Aggie ice cream.

Image 1. A Photograph of Erin Young, the Author.

*Photograph of Erin Young taken on Stanford University
Campus July 2, 2009.*

