PFK-1 Transcript Amounts in the Liver and Skeletal Muscle of Pigeons and Quail Acutely Exposed to High Embryonic Incubation Temperatures

Mike Loveless

Loveless.40@osu.edu

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Project Advisor:

Dr. Pasha Lyvers Peffer

Department of Animal Sciences

Lyvers-peffer.1@osu.edu

Abstract

The developing avian embryo is reliant on external sources of heat. Incubation temperatures above or below optimal alter development and metabolic function, and may compromise emergence and post-hatch adaptation. Studies in chick embryos indicate that high incubation temperature increases glycolysis and reduces hepatic glycogen needed during hatching. As a key glycolytic enzyme, phosphofructokinase-1 (PFK-1) is a likely target for the temperature-induced effects on pathways of glucose metabolism. However, there is limited data characterizing PFK-1 in avians exposed to high incubation temperatures. This study used real time PCR to compare PFK-1 mRNA transcript amounts within the breast muscle and liver tissue of the precocial japanese quail (*Coturnix japonica*) and the semi-altricial domestic pigeon (*Columbia livia*). Tissue samples were collected previously from birds exposed to increased incubation temperatures (40.8˚ for 3 hours during embryonic d 10 and 11 for quail, and d 13 and 14 for pigeon, equivalent to Hamburg and Hamilton stages 39 and 40) or a control temperature (37.6˚ C throughout incubation). Total RNA was isolated, reverse transcribed, and cDNA was pooled by species and heat treatment for analysis. High incubation temperatures resulted in a down regulation in PFK-1 transcript amounts in both liver and breast muscle in pigeons. Transcript amounts were 50 and 2.78 fold greater for liver and breast muscle, respectively, for birds incubated under control temperatures compared to high heat. Similarly, PFK-1 transcript amounts in quail liver of birds exposed to high incubation temperatures was 80% the value of the control, but breast muscle PFK-1 transcript amounts increased to 165%. Findings suggest unique effects of incubation temperature for precocial compared to altricial species.

Introduction

The chicken egg has been used for much of the research done to date on avian embryonic development. Embryonic development in avian species is unique as their development occurs outside of the maternal environment. There are studies that describe differences across avian species in developmental patterns such as metabolic rate or incubation length (Vleck, et al., 1979; Deeming and Pike, 2013), but Vleck and Vleck (1987) states that the observed differences are due to unique timing of development rather than different processes. Thus, the general stages of avian embryonic development are conserved.

The first stage is characterized by full germ development, the second by embryo completion, and the final third by preparation for hatch and emergence. During the first stage of development, the chorioallantois is not fully formed within the egg, so there is limited transfer of oxygen to the embryo. This prohibits aerobic metabolism of the lipids found within the yolk. Instead, the embryo uses anaerobic glycolysis of available glucose as the main source of energy during this time (Moran, 2007). The lactate produced by this process is recycled through the cori cycle in the liver (De Oliveira, 2008). Anaerobic glycolysis is supported by the presence of enzymes involved in degradation of glucose in most avian embryonic tissues from the earliest days in ovo (Hazelwood, 1986).

Upon entering the second stage, the chorioallantois is completed, and the embryo switches to utilizing lipids as its main energy source. Carbohydrates begin to be stored as glycogen in the liver and muscle tissues (Moran, 2007). Because of this, lipid metabolic pathways are most important from after the first week of incubation, until the last two or three days before internal pipping (De Oliveira, 2008). There is little carbohydrate stored in the egg initially, but Hazelwood (1986) found that gluconeogenic enzymes tend to increase in activity from beginning of embryonic development to a peak at 10-20 days post hatch. This increase occurs as the embryo utilizes gluconeogenesis to build glycogen stores that will be used during emergence.

Approaching emergence and the third stage, the embryo ruptures the stored albumin in the egg which mixes with the amniotic fluid. This mixture is absorbed through the embryonic gut, and is made possible by the initial villi in the small intestine. These villi are capable of macromolecule absorption, similar to what is observed in mammals with colostrum absorption. Additionally, antitrypsin factors are present and prevent digestion of the mixture in the gut so that it can be absorbed whole (Moran, 2007). The embryo uses the absorbed albumin proteins in gluconeogenesis resulting in raised blood glucose levels that support glycogen storage in hatching muscles and the liver. Once this fluid is fully absorbed, the third stage begins, marked by internal pipping piercing into the air cell.

The previously stored glycogen is utilized in part by glycolysis in hatching muscles which are exclusively anaerobic (Moran, 2007). Freeman (1969) states that a rapid decrease in liver glycogen at the end of incubation, days 19 and 20, coincides with an increase in plasma glucose, and suggests that this increase in blood glucose is for use by the central nervous system.

Hatching muscles and the central nervous system depend on energy stored in glycogen, illustrating the importance of glycolysis at the end of the emergence stage.

Glycolysis is the metabolic process which converts a single glucose molecule into two pyruvate molecules which can be utilized for energy production. Phosphofructokinase-1 (PFK-1) converts fructose-6-phosphate into fructose-1,6-bisphosphate, and it is the first step in the glycolytic mechanism where the products are fully committed to going through glycolysis. Because it is the step which locks the cell into glycolysis, the PFK-1 reaction is highly regulated by molecules indicative of high energy in the cell, such as ATP and Citrate. If the cell has high concentrations of these molecules, the feedback loop decreases PFK-1 activity to decrease glycolysis. Due to its important role in committing the cell to the glycolytic mechanism, this enzyme is key to determining the prevalence of glycolysis. Indeed, Blomstrand, et al. (1983) utilized PFK-1 as an indicator for glycolytic activity in the pectoral muscle of pigeon and chicken. Their study revealed differences in PFK-1 activity rates between the species, indicating that PFK-1 activity is variable across different species of avians. This variability doesn't imply other enzyme activity in its place however, as it has been shown that PFK-1 is the leading enzyme for phosphorylating fructose-6-phosphate in both pigeon and chicken, so it is still a valid marker of glycolysis (Opie and Newsholme, 1966).

Differences exist in embryonic development patterns across avian species such as metabolic rate and weight gain, and some of these differences can be attributed to the maturity of the organism. Precocial organisms, such as the chicken or the japanese quail, hatch at a more developed stage, while altricial species require more maturation after hatch. The domestic pigeon is a semi-altricial species. Vleck, et al. (1980) found that precocial metabolic rate increases exponentially until 80% of the way through incubation where it plateaus until hatch. Altricial metabolic rate increased continuously throughout the entire incubation period. Weight gain followed the same trend in both species types. Precocial species have a higher energy requirement during embryonic growth, which is met by increased amounts of yolk, 35% of egg weight compared to 20% in altricial species (Ricklefs, 1977), and higher concentration of lipids within the yolk (Vleck et al, 1980). There is debate over the presence of a plateau in altricial species. Prinzinger and Dietz (1995) state that a plateau does exist in altricial species, but the amplitude and duration is reduced compared to what occurs in precocial species. Developmental differences observed in between the species types are likely a result of difference in timing rather than difference in process. Consequently, energetics and fuel use are similar between altricial and precocial birds, but occurring at different points in time (Vleck and Vleck, 1987).

Studies reveal that the embryonic environment can greatly impact the organism post hatch. External influences such as light exposure, audible cues from parents, and temperature all can contribute to egg development (Reed and Clark, 2011). In a natural setting, the parents can impact these conditions. For example the egg's temperature is maintained by its mother sitting on the nest. In a production or research setting, temperature is maintained by an incubator. Willemsen, et al. (2010) found that chickens exposed to higher than normal incubation temperatures from embryonic days 16 to 18.5 saw increased embryonic mortality, increased duration of emergence, and decreased chick weight at hatch. Additionally, an increased incubating temperature led to a 30% decrease in hepatic glycogen stores taken on embryonic day 18. Another study exposed chickens to increased incubation temperatures from embryonic day 10.5 to hatch and also found a loss of hepatic glycogen, and determined that it was due to increased glucose oxidation rather than decreased glycogen synthesis, and may be a cause of

reduced hatchability (Molenaar, et al., 2013). These effects are the results of environmental changes during embryonic development, and exemplify the importance of external influences on growing birds.

One characteristic of note in the Willemsen, et al. (2010) and Molenaar, et al. (2013) studies is the difference in when they applied increased incubation temperature. Conditions experienced by an embryo during critical periods of development, such as the time approaching emergence, may determine the control points for the feedback loops regulating bodily systems for the animal's entire life (Tzschentke and Plagemann, 2006). Critical periods of development likely occur at different times between precocial and altricial species given the distinct growing patterns they follow. Thus, the species may be unequally affected by environmental influences during an identical period of incubation. A previous part to this study found that acute exposure to increased incubation temperatures during equivalent stages of development between precocial and semi-altricial species resulted in a reduction in liver weight in the semi-altricial species, but not the precocial species (*unpublished*).

There is increasing evidence that changes in the environment during embryonic development can affect not only an embryo's ability to survive, but its performance post hatch. Research has been done showing the effects of incubation temperatures on hatchability, embryo mortality, chick weight, and incubation duration. Studies also note that changes in glucose flux occur as demonstrated by decreased hepatic glycogen levels in chickens exposed to increased incubation temperature (Willemsen, et al., 2010), and that those changes are due to increased glucose oxidation (Molenaar, et al., 2013). Increased glucose oxidation should be supported by increased expression of glycolytic enzymes. PFK-1 commits the cell toward glycolysis rather than glycogen synthesis (Hazelwood, 1986), and is a valid target for gaining insight into glycolytic activity in tissues. This study investigated the mRNA transcript levels of PFK-1 in breast muscle and liver tissue of precocial japanese quail and semi-altricial domestic pigeon in order to determine the effect of acute elevation of incubation temperature during embryonic development on a key regulator of glycolysis.

Procedures and Methodologies

Procedures were performed using preserved breast muscle and liver tissue samples collected in a previous study. Briefly, fertilized quail and pigeon eggs were allotted to control temperature or high temperature treatment groups. Control eggs were incubated at 37.6˚ (±0.01) with an average relative humidity of 56% (±0.31) for the duration of incubation. The high treatment group was initially incubated at 37.6˚ (±0.01) with an average relative humidity of 56% (±0.31). During embryonic ages 13 and 14 days for pigeon, and 10 and 11 days for quail (equivalent to Hamburg and Hamilton stages 39 and 40), high treatment eggs were temporarily transferred to another incubator. The high treatment group was incubated at 40.8˚C (±0.10) with an average relative humidity of 56% (±0.49) for 3 hours. After incubation at 40.8˚ C, high treatment groups were returned to the original incubator and incubating conditions. Prior to transferring to the high treatment incubator, on day 8 of incubation, eggs were candled and dead embryos or clear eggs were removed. On day 16 of incubation all eggs were transferred to a tabletop incubator at 37.8˚ (±0.09) with an average relative humidity of 73% (±0.004) for hatching. Chicks that emerged were euthanized and liver and breast muscle without rib was removed and stored at -20˚C in an RNAlater solution.

RNA was isolated using the TRI reagent (Sigma). RNA integrity was verified by agarose gel electrophoresis using ethidium bromide staining, and RNA quantity and purity was assessed using spectrophotometric analysis (Nanodrop 1000; Thermo Fisher Scientific). Isolated RNA was reverse transcribed using Omniscript reverse transcriptase (Qiagen) and a mixture of random hexamer primers and oligoDT primers. Each reaction contained 1 ug of RNA within a final reaction volume of 20 uL.

Real Time PCR was performed using the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems) and SYBR® Green (Qiagen) fluorescence was used for cDNA detection. cDNA was pooled by tissue and species. Intron spanning primers for RPL 13 (forward: TTTCAAACGGGAGAAGGCCC and reverse: CCAAAGAGACGAGCGTTTGC), and PFK-1 muscle (forward: CCTGGTAGCCCTCATGCAC and reverse: CCATCGCTGTCCTCACCTC) were used from a previous study. Intron spanning primers for PFK-1 liver (forward: TAGGCACCAAGCGGACTCTG and reverse: ATGCCTCAAAGCCGCCAATG) were designed from published NCBI chicken sequences (Accession number: NM_001318442.1) using Primer3 software. Optimal annealing temperature was determined experimentally for each primer set (58.1˚C for RPL 13, PFK-1 muscle, and PFK-1 liver). Following DNA polymerase activation at 94˚C for 15:00 minutes, cycling parameters were 94˚C for 15 seconds, 58.1˚C for 30 seconds, followed by 72˚C for 30 seconds for 45 cycles. Fluorescence data was collected at the end of the elongation cycle and a dissociation curve was programmed for detection of non-specific amplification products. Samples were run in triplicate and negative controls (minus template) were included on each plate. The final reaction volume used was 25 uL including forward and reverse primers, each at a concentration of 0.3 uM, and 1 uL of pooled cDNA.

Relative transcript amounts were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001) and normalized to RPL 13. Results are presented as fold differences in transcript expression between control and high groups.

Results

In pigeon, transcript abundance decreased in response to high incubation temperatures by 50 fold in liver and 2.78 fold in muscle (Figure 1A). Transcript amounts in quail liver also decreased to 80% of the control value when birds were exposed to high incubation temperatures but increased to 165% in quail muscle (Figure 1B).

Figures

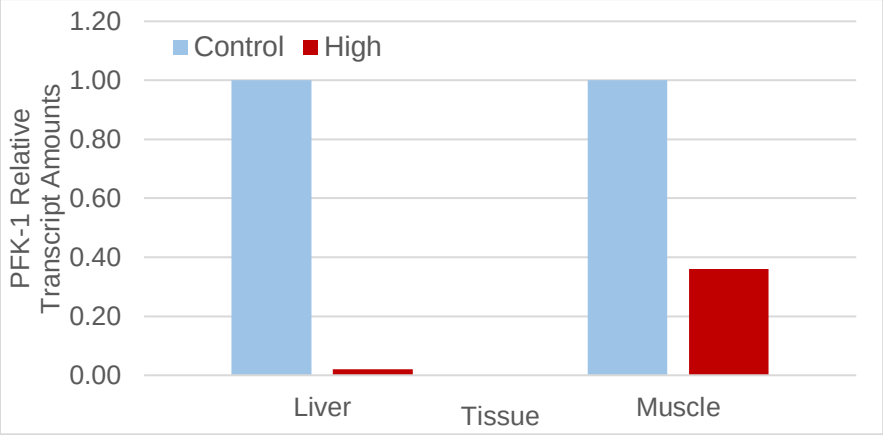

Figure 1A

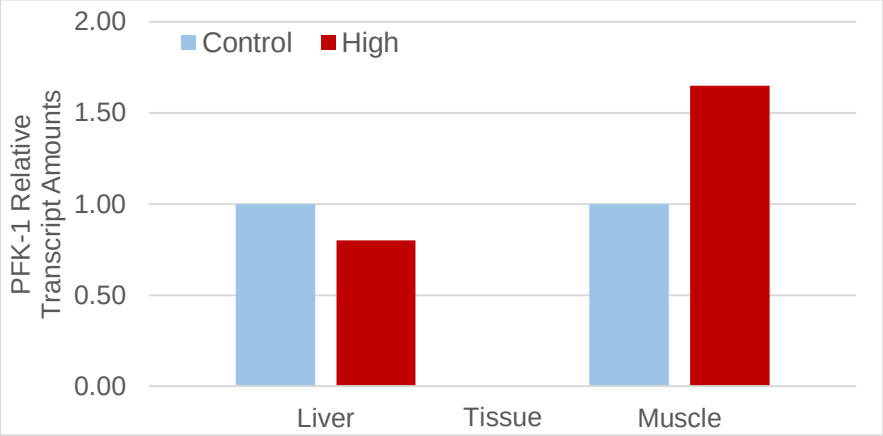

Figure 1B

Discussion

PFK-1 is the committal step for glycolysis, and thus its activity is highly regulated. PFK-1 has multiple allosteric regulators that respond to the energy condition in the cell and include citrate, fructose-2,6-bisphosphate, and AMP/ATP. Various isoforms of PFK-1 exist throughout the animal body (Somero, et al., 1991). Kahn, et al. (1979) defines the isoforms in humans as muscle type, fibroblast type, and liver type. There are hybrid forms made up of combinations of isoforms across different tissues, but some tissues exhibit consistent isoform use, including muscle tissue and liver tissue, which incorporate muscle and liver type PFK-1 respectively. These tissue specific isoforms differ in their inhibition by ATP, which is less prevalent in muscle type than liver type PFK-1 (Kahn, et al., 1979).

Isoform prevalence across tissues is a reflection of the different biological roles of the tissue. Liver is generally a gluconeogenic organ while muscle is generally a glycolytic organ. Liver type PFK-1 is inhibited more strongly by ATP, showing that in a positive energy state the liver focuses less on continued ATP production and more on energy storage. Muscle type PFK-1 is significantly less inhibited by ATP, demonstrating that regardless of energy condition of the cell, muscle tissue is focused on energy production. Because the liver has a limited capacity for

energy storage, glucose is directed toward muscle where it is partitioned between storage and use through glycolysis.

The increase of PFK-1 in muscle tissue of quail, and the decrease in liver tissue PFK-1 observed in this study coincide with the increase in glucose oxidation observed by Molenaar, et al. (2013). While glucose oxidation was not measured in this study, the observed changes for the precocial quail suggest that increased incubation temperatures may reduce the livers capacity for glucose oxidation, redirecting glucose toward muscle tissue for oxidation. For the semi-altricial pigeon exposed to an acute increase in incubation temperature, PFK-1 transcript amounts in both the liver and muscle tissues decreased. A previous study (*unpublished*) also showed a significant decrease in liver weight post-hatch for pigeons exposed to high incubation temperatures, which was not observed in quail. Collectively, these findings reveal distinct differences between altricial and precocial birds exposed to acute high incubation temperatures.

Summary

The study of external influences on embryonic development in avians continues to be a valuable arena for research. Our findings revealed that acute exposure to increased incubation temperatures can impact PFK-1 transcript amounts in liver and breast muscle, suggesting an effect on glucose oxidation. However, knowledge can still be gained on how different initiation time and duration of an environmental change, such as temperature, can have different impacts on an embryo. More research could also elucidate the variation of these impacts between precocial and altricial species, which follow different rates of embryonic development.

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