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# Voluntary Exercise in Phosphorylase Kinase Deficient Mice

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# VOLUNTARY EXERCISE IN PHOSPHORYLASE KINASE DEFICIENT MICE

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Sciences with

Honors Graduate Distinction at Western Kentucky University

By

Ashley M. Mefford

\* \* \* \* \*

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2010

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2010

## ABSTRACT

Phosphorylase kinase (PhK), a key regulator of glycogenolysis, is critical for maintaining blood glucose levels thus providing energy to sustain muscle contraction. A deficiency of PhK in skeletal muscle is the cause of one type of glycogen storage disease (GSD) in humans. This study investigates the physiological and genetic adaptations that occur in a mouse model of GSD, I/LnJ mice, in response to voluntary exercise. Juvenile (6-8 weeks old) and adult (12-14 weeks old) I/LnJ and wild-type C57/Bl6 mice exercised voluntarily for 1, 2 or 5 weeks. Exercise data was calculated as mean daily running time, daily running distance, total running time, total running distance, and average speed. After five weeks, adult and juvenile I/LnJ mice were running 45-70% of the daily distance of age-matched, wild-type mice. A training effect was observed in wild-type mice during the five week exercise period, but no significant difference was observed in heart/body weight ratios in exercised mice compared to non-exercised controls. Expression levels of glucose transporter 4 (GLUT4), pyruvate dehydrogenase (PDHA1), and phosphofructokinase (PFKM) as a result of exercise were determined by quantitative RT-PCR in both I/LnJ and



C57/Bl6 mice. No significant differences in expression levels were found between mouse strains. Our long term goal is to gain insights into the I/LnJ strain's PhK deficiency in order to better understand human GSDs.

Keywords: exercise, phosphorylase kinase, PhK, glycogenoses, mice, I strain

Dedicated to David & Mae Mefford and James & Roberta Mefford for their constant support during my pursuit of higher education and success in life.

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## CHAPTER 1

### INTRODUCTION

One of the common sources of energy in the body is the simple sugar glucose, which is acquired from dietary carbohydrates or can be synthesized in the body from various other substrates (Figure 1.1). To be used as energy, glucose is transported to cells through the blood stream where it is broken down via a process called glycolysis to produce pyruvic acid. The irreversible and path-determining step in glycolysis is the conversion of fructose 6-phosphate, a derivative of glucose, into fructose 1,6-bisphosphate, catalyzed by phosphofructokinase (1). The resulting pyruvic acid is converted by pyruvate dehydrogenase into acetyl-CoA, which is sent into the citric acid cycle to produce a functional energy molecule, adenosine triphosphate (ATP) (1). ATP is referred to as the energy currency of the cell and is used to drive countless cellular processes including muscle contraction.

For storage purposes, glucose can be converted into a polymeric form, glycogen, which is mostly found in skeletal muscle tissue and liver tissue, although other cells can synthesize it (2). When needed, glycogen is broken down through the process of glycogenolysis into glucose molecules in order to

## Dietary Carbohydrates

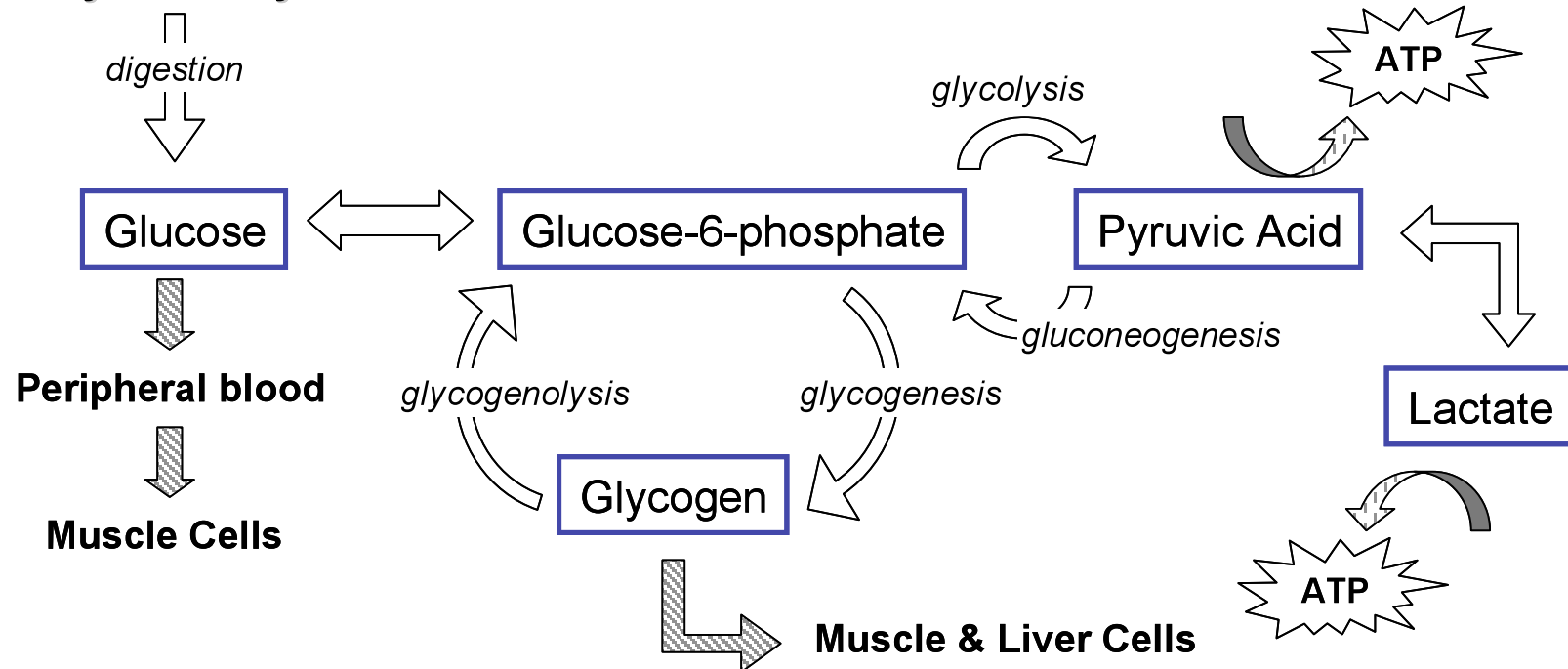


Figure 1.1. **Metabolism of dietary carbohydrates.** As carbohydrates are digested and converted into glucose-6-phosphate, their fate is determined by the needs of the body and complex signaling mechanisms.



sustain muscle contraction and to maintain blood glucose levels (3). Two key hormones involved in the regulation of glycogenolysis are epinephrine, aka, adrenaline, which acts on muscle and liver cells, and glucagon, which mainly affects liver cells (4). Hormonal regulation is crucial, for instance, when glucose levels in the blood are too low; the pancreas is stimulated to secrete glucagon which binds to specific receptors on the liver to initiate glycogenolysis (5). Several enzymes are involved in the reaction mechanism of glycogenolysis (Figure 1.2). The rate limiting step of the reaction is catalyzed by glycogen phosphorylase, which severs the glucose molecules via a phosphorylation mechanism. Glycogen phosphorylase is likewise activated via phosphorylation by phosphorylase kinase (PhK) (6).

PhK is a hexadecamer made up of four sets of four different subunits,  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ . The  $\alpha$ - and  $\beta$ - subunits are thought to be mainly regulatory, whereas the  $\gamma$  subunit is the catalytic subunit of the enzyme (7). The activity of the enzyme is dependent upon  $\text{Ca}^{2+}$  as its  $\delta$  subunit is an intrinsic calmodulin molecule. In skeletal muscle cells, contraction is stimulated by an influx of  $\text{Ca}^{2+}$  into the cytoplasm which also stimulates PhK activity. Therefore, muscle contraction and glycogenolysis are linked (6). Muscle contraction is sustained through  $\text{Ca}^{2+}$ -stimulated glycogenolysis as more glucose becomes available for ATP production.

In conjunction with internal glycogen stores, the peripheral blood is also a large source of glucose for skeletal muscle. Uptake of glucose from the blood

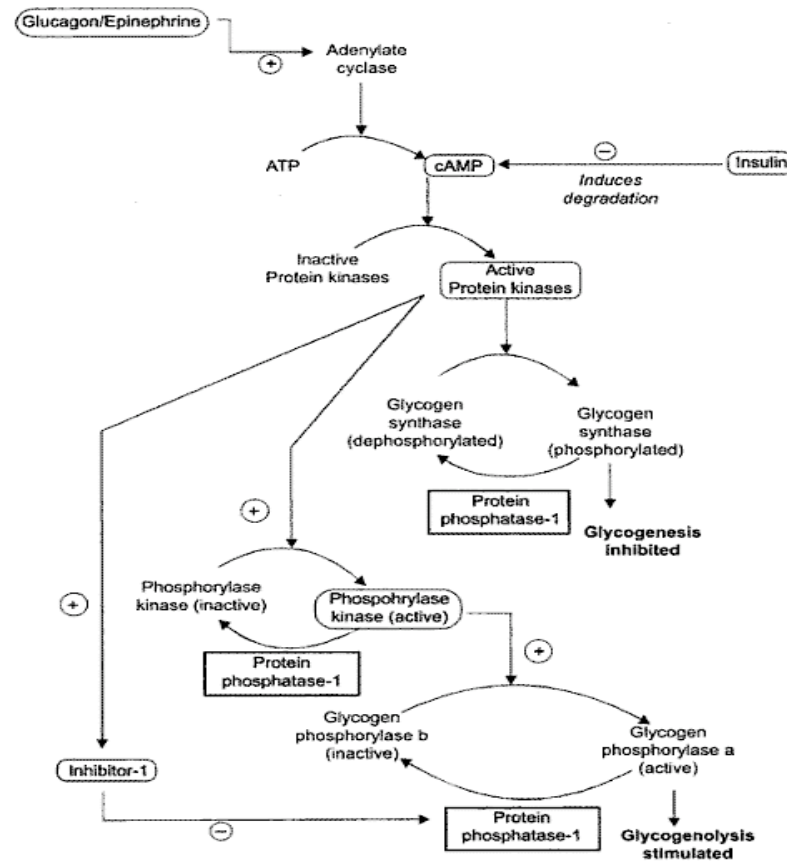


Figure 1.2. **Hormonal control of glycogenolysis.** Source: Puri, Dinesh (2006) in *Textbook of Medical Biochemistry*, 2<sup>nd</sup> Ed., Elsevier, New Delhi.

stream occurs via glucose transporters. Skeletal muscle absorbs glucose mainly via glucose transporter 4 (8, 9). During exercise these transporters migrate from the cytoplasm to the plasma membrane in order to function. Unlike in resting muscle, the movement of these transporters is not a function of the hormone insulin. Rather, the movement is believed to be influenced by  $\text{Ca}^{2+}$  (10). The mechanism by which  $\text{Ca}^{2+}$  stimulates this movement has not been fully elucidated. Evidence suggests that contraction-related pathways involving protein kinase C (PKC) and adenosine monophosphate kinase (AMPK) pathways may be used (11, 12). Since the levels of glucose in the blood are maintained by the breakdown of glycogen in the liver, hepatic glycogenolysis may be as important for sustaining muscle contraction during activity as skeletal muscle glycogenolysis (13).

Numerous diseases are associated with abnormalities in different enzymes involved in the regulation and utilization of glycogen. Collectively, these diseases are referred to as glycogen storage diseases (GSD) or glycogenoses (14). Such diseases are identifiable in humans via genetic testing as well as other mammals such as rats and mice. The cause of each type of GSD is unique, and most are not yet fully understood. One deficiency that has been under investigation for some time is the PhK deficiency observed in I strain (I/LnJ) mice (15). The PhK deficiency found in these mice affects their ability to breakdown glycogen in skeletal muscle (16, 17). PhK activity has been measured to be 0.2-0.3% of normal in the limb and back muscles of these mice

and 1.5% of normal in the soleus muscle (15, 16, 18). It has also been determined that the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of PhK are absent in the skeletal muscle (16, 19). A mutation in the muscle specific  $\alpha$ -subunit of the enzyme (PHKA1) is the suggested cause of the deficiency and absence of all three subunits (20, 21).

In humans, skeletal muscle PhK deficiencies occur much less frequently than hepatic PhK deficiencies. However, there are well documented examples in human patients. Several patients, who were confirmed to have skeletal muscle PhK deficiencies via genetic testing, presented with muscle weakness and rapid fatigue upon exercise (22, 23). Other dominant symptoms included muscle cramps or muscle stiffness combined with exercise intolerance (24, 25, 26).

Rodent glycogenoses provide animal models for studying the causes of such diseases in order to gain insight into the human disease. The goal of this particular research project was to explore the physiological and metabolic adaptations that occur as a result of voluntary exercise in the PhK deficient I strain mice. Given the symptoms presented in human patients, it was predicted that mice with a similar deficiency would likely endure less voluntary exercise likely due to muscle weakness and cramping. Since it is likely that the I strain mice have to utilize sources of energy other than glycogen in order to maintain activity, it is expected that the expression levels of several key enzymes involved in carbohydrate metabolism will be affected by the deficiency.

## CHAPTER 2

### MATERIALS AND METHODS

#### Exercise Study

The mice used in this study were wild type C57/Bl6 and PhK-deficient I/LnJ mice, also called I strain mice. Mice were housed in an IACUC approved facility with water and food provided *ad libitum*. Both adult mice and juvenile mice were tested at 12-14 weeks and 6-8 weeks of age, respectively. Each mouse was placed in a separate cage with a standard 6 inch hamster wheel equipped with a Sigma<sup>TM</sup> speedometer (Figure 2.1). Each speedometer was set to a wheel 3672 units (as consistent with the speedometer's programming) in circumference to increase the sensitivity of the sensor. Upon analysis, the data were corrected for the actual circumference of the wheels, approximately 459 mm. This circumference is an estimate since all the wheels vary slightly on the millimeter scale.

The mice were allowed to voluntarily exercise on the hamster wheels for a one, two, or five week exercise period. The speedometers recorded the daily time, daily distance, and total distance each mouse ran throughout the exercise period. The data from the computers were collected every 48 to 72 hours until

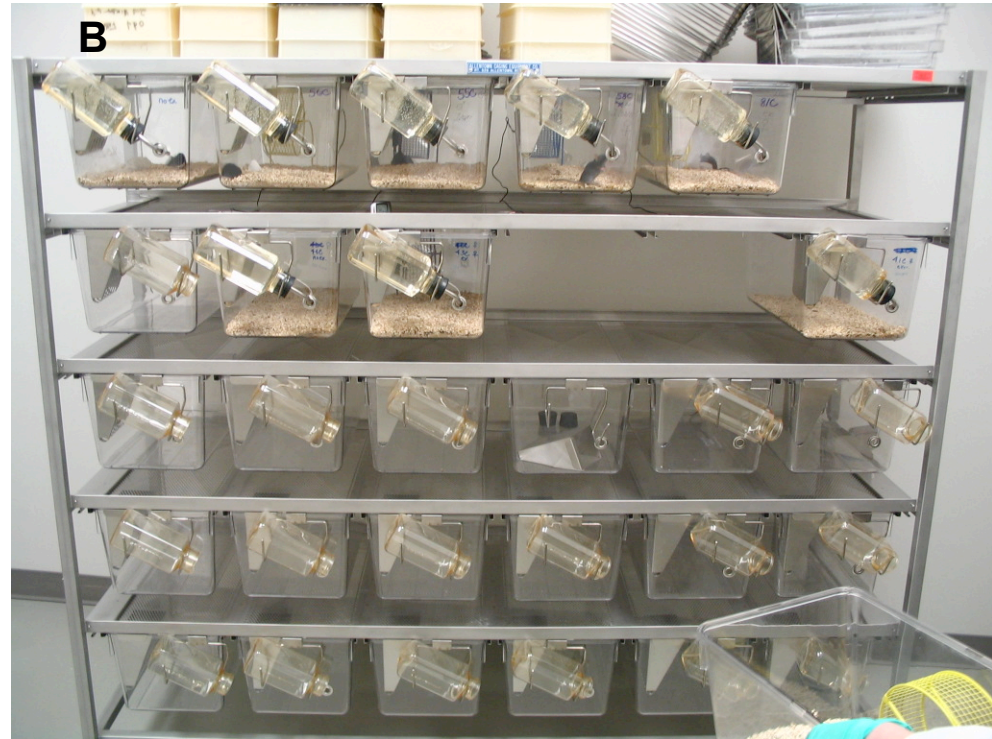
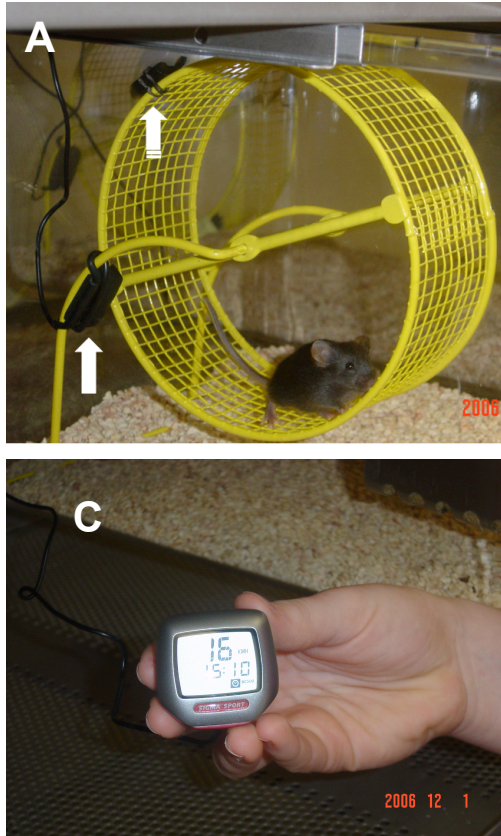
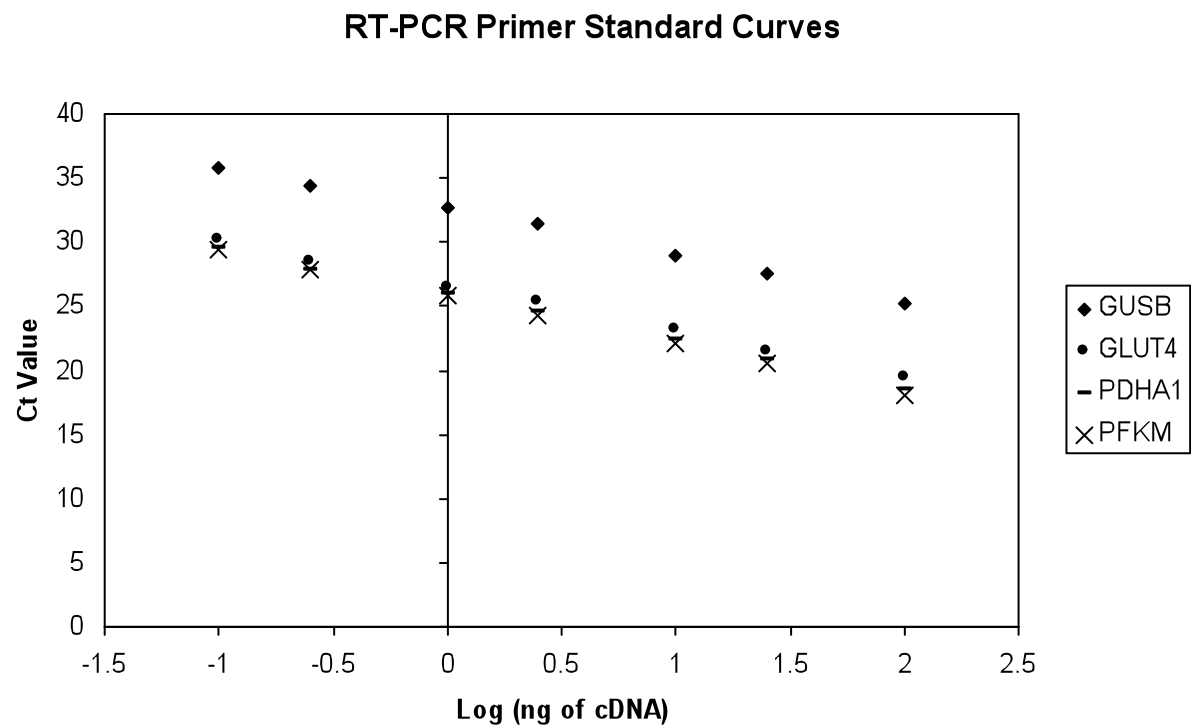


Figure 2.1. **Exercise study, equipment and set up.** A: C57/Bl6 mouse running on 6 inch hamster wheel. Solid arrow = sensor. Broken arrow = magnet attached to wheel. B: Mouse housing facility. C: Sigma™ speedometer.

the completion of the exercise period and used to calculate average daily running time and distance, as well as average running speeds. Between two and ten biological replicates were tested for each experimental group according to the amount of mice available. Occasionally, data was unable to be collected for the adult mice since they were prone to chew through the speedometer wires. The data for the biological replicates were averaged with outliers, any data point two standard deviations or more from the mean, being removed. Statistical analyses were performed in the form of unpaired t-tests to determine significant differences between the activity of the wild type and I strain mice as well as any increases in activity over time. At the end of the exercise period each mouse was euthanized. The body mass and heart mass of each mouse was quantified to observe anatomical adaptations to the exercise.

#### Gene Expression Assays

Gross muscle samples were taken from the left hind limb of each mouse to be used for gene expression studies. Total RNA was isolated from the muscle samples using a RNeasy Fibrous Tissue Mini kit [Qiagen]. The RNA was converted to cDNA with ABI High Capacity cDNA kit according to the manufacturer's protocols [Applied Biosystems]. Expression levels of three genes were quantified including: glucose transporter 4 (GLUT4), pyruvate dehydrogenase (PDHA1), and phosphofructokinase (PFKM) to observe adaptations that may occur in crucial metabolic enzymes (see Chapter 1) as a



**Figure 2.2. RT-PCR primer standard curves.** A standard curve of pooled cDNA assayed at concentrations of 0.1, 0.25, 1.0, 2.5, 10, 25, 100 ng with each primer as described in "Materials and Methods." GUSB =  $\beta$ -glucuronidase; GLUT4 = glucose transporter 4; PDHA1 = pyruvate dehydrogenase; PFKM = phosphofructokinase.  $R^2$  values: GUSB = 0.996, GLUT4 = 0.998, PDHA1 = 0.999, and PFKM = 0.999.



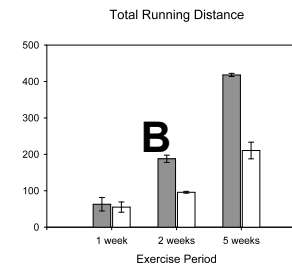
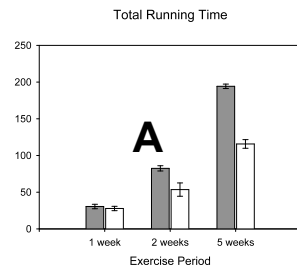
response to the PhK deficiency. The primers used to probe for these genes were Taqman Gene Expression Assays purchased from inventory at Applied Biosystems. Each primer was carefully selected to include regions that cross exon boundaries. Expression was quantified using real-time PCR techniques in triplicate with a total of three biological replicates each. In each reaction, 8 ng of cDNA was used. Relative expression was determined from the  $C_t$  values which represent the cycle at which each sample reached a manually-set threshold of amplification for the target gene. The  $C_t$  values were normalized to an endogenous control,  $\beta$ -glucuronidase (Gusb), a gene unrelated to the metabolic pathways under examination. A relative standard curve analysis was used to correct  $C_t$  values, accounting for small variations in primer efficiency. Standard curve samples were made by pooling cDNA from one biological replicate of each experimental group and assaying concentrations ranging from 0.1 ng to 100 ng. Standard curves for all four primers used can be seen in Figure 2.2.

## CHAPTER 3

### RESULTS

The cumulative time and distance run by the juvenile mice during the exercise period are shown in Figure 3.1. After two weeks of voluntary exercise, wild-type mice ran both significantly more cumulative time and distance than the age-matched I strain mice. Following five weeks of exercise, the I strain mice ran  $116 \pm 6$  hours and  $211 \pm 23$  km. Wild-type mice ran an average of  $194 \pm 3$  hours and  $418 \pm 4$  km, approximately twice as much as the I strain mice.

In the adult mice few significant differences were found in daily activity between strains except the daily amount of time run after two weeks, where wild-type mice ran  $375 \pm 15$  min/day and the I strain ran  $268 \pm 64$  min/day (Figure 3.2). This was not the case in juvenile mice. After five weeks of exercise, the juvenile wild-type mice ran for significantly longer lengths of time on a daily basis at  $349 \pm 15$  min/day than the I strain mice at  $219 \pm 6$  min/day. Juvenile wild-type mice also ran significantly farther than I strain mice daily, an average of  $15 \pm 2$  km/day compared to the I strain mice that ran  $7 \pm 1$  km/day. These data suggest that the I strain mice were unable to increase their tolerance during the exercise period and did not undergo the training affect observed in the wild-type



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Figure 3.1. **Cumulative time and distance.** The total amount of time (A) and distance (B) run by juvenile mice is shown. Values are given as averages with error bars representing standard error. Wild type = dark bars; I strain = white bars. n = 2-10. \*,  $p < 0.05$  compared to age-matched mice of the opposite strain.

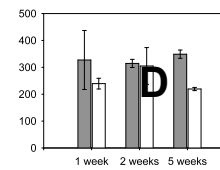
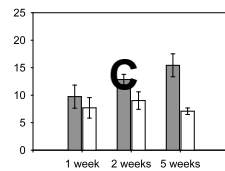
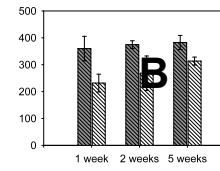
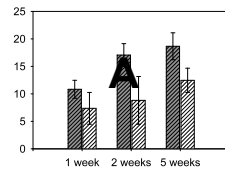
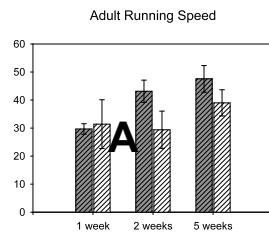


Figure 3.2. **Average daily activity.** The average daily activity of adult (*A*, *B*) and juvenile (*C*, *D*) mice is shown. Daily distances in kilometers (*A*, *C*) and daily times in minutes (*B*, *D*) are given as averages with error bars representing standard error. Adult = striped bars; Juvenile = solid bars; Wild type = dark bars; I strain = white bars.  $n = 2-10$ . \*,  $p < 0.05$  compared to age-matched mice of the opposite strain. #,  $p < 0.05$  compared to 1 week of same strain.

mice.

These daily activity levels are comparable to those reported by Allen, *et al.* (2001) in an exercise study done of C57/Bl6 mice. In this study, mice 8-10 weeks old ran between 3.1 and 16.2 km/day. The adult wild-type mice in our study ran a higher maximum of 18.6 km/day after five weeks, but otherwise all of the wild-type mice data fall into this range. It was also reported that the mice in Allen, *et al.* (2001) ran between 2.4 and 7.1 hours per day which is equivalent to 144-426 min/day. The averages found in our study for the juvenile (6-8 weeks) and adult mice (10-12 weeks) fall within this range (Figure 3.2).

Interestingly, both juvenile and adult mice of each strain ran approximately the same speed as shown in Figure 3.3. After five weeks, wild-type and I strain juvenile mice were running  $43 \pm 4$  m/min and  $32 \pm 3$  m/min respectively, whereas adult wild-type and I strain mice ran  $48 \pm 5$  m/min and  $39 \pm 5$  m/min after five weeks of acclimation to the exercise. After statistical analysis by t-test, these speeds were determined to be not significantly different. Therefore, I strain mice were on average able to run as fast as healthy wild-type mice. It is notable that these averages are higher on average than those reported elsewhere for C57/Bl6. The range of speeds for the juvenile mice in the present study measured 22.4 m/min-63.2 m/min compared to an average of 26.4 m/min and maximum of 44.8 m/min reported by Allen, *et al.* (2001). This is an expected discrepancy given that the maximum km/day data is also higher in the present study, but the min/day data between the two studies are consistent.



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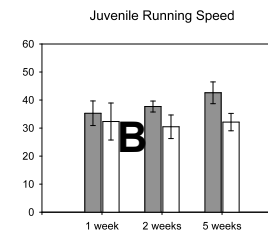


Figure 3.3. **Average running speed.** The average speeds run by adult (*A*) and juvenile (*B*) mice are shown. Values are given as averages with error bars representing standard error. Wild type = dark bars; I strain = white bars.  $n = 2-10$ . \*,  $p < 0.05$  compared to age-matched mice of the opposite strain. #,  $p < 0.05$  compared to 1 week of same strain.

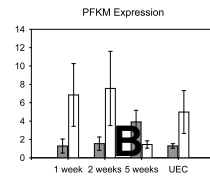
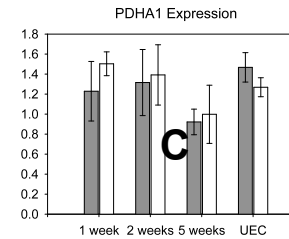
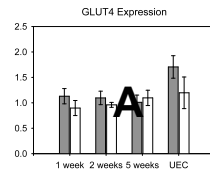
Heart mass/body mass (mg/g) ratios were determined (Table 1) in order to assess physiological hypertrophy that may have resulted from the exercise. Compared to the unexercised controls, no significant differences were found in the wild-type or I strain exercised mice, indicating a lack of cardiac hypertrophy. Furthermore, no significant increases in the heart mass/body mass ratios were observed among any of the experimental groups as the length of the exercise period increased.

The observation of physiological adaptations was followed by a study of the metabolic adaptations in these mice by gene expression assays (Figure 3.4). The relative expressions of mRNA for glucose transporter type 4 (GLUT4), phosphofructokinase (PFKM), and pyruvate dehydrogenase (PDHA1) are shown for the juvenile wild-type and I strain mice only. The singularly significant difference determined among the expressions levels was that of PDHA1 in wild-type five-week mice versus the unexercised wild-type control. Comparing the I strain mice to wild-type, there were no significant differences between the expression levels of any of the three genes tested. It is possible that the lack of significance is due to the high variability among biological replicates, which would also suggest that the set of genes observed was not affected in a consistent manner due to the PhK deficiency.

|          |                 | Wild Type   |             |              |                     | I strain   |             |              |                     |
|----------|-----------------|-------------|-------------|--------------|---------------------|------------|-------------|--------------|---------------------|
|          |                 | One Week    | Two Weeks   | Five Weeks   | Unexercised Control | One Week   | Two Weeks   | Five Weeks   | Unexercised Control |
| Juvenile | Heart Mass (mg) | 90.0 ± 5.8  | 106.0 ± 6.8 | 122.6 ± 6.0  | 116.7 ± 14.5        | 97.7 ± 1.9 | 105.6*      | 116.4 ± 3.8  | 125.0 ± 7.7         |
|          | Body Mass (g)   | 16.5 ± 0.6  | 18.1 ± .8   | 18.9 ± 1.1   | 19.0 ± 1.8          | 15.9 ± 0.6 | 18.0*       | 20.2 ± 0.9   | 22.0 ± 1.3          |
|          | HM/BM           | 5.4 ± 0.3   | 5.9 ± 0.5   | 6.5 ± 0.1    | 6.1 ± 0.2           | 6.2 ± 0.3  | 5.9*        | 5.8 ± 0.2    | 5.7 ± 0.03          |
| Adult    | Heart Mass (mg) | 146.9 ± 8.5 | 133.7 ± 8.7 | 138.5 ± 17.0 | 131.6 ± 5.2         | 120.6*     | 124.4 ± 6.4 | 132.5 ± 12.4 | 126.8*              |
|          | Body Mass (g)   | 23.7 ± 0.7  | 23.2 ± 0.8  | 23.4 ± 1.3   | 23.5 ± 1.6          | 18.8*      | 20.1 ± 0.5  | 21.3 ± 1.8   | 20.7*               |
|          | HM/BM           | 6.2 ± 0.6   | 5.7 ± 0.2   | 5.9 ± 0.6    | 5.7 ± 0.4           | 6.4*       | 6.2 ± 0.3   | 6.2 ± 0.1    | 6.1*                |

Table 1. **Heart mass/body mass ratios.** Values are given as averages ± standard error. n = 2-10. \*Data points are the average of two mice.





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**Figure 3.4. Gene expression.** The relative expressions of GLUT4 (A), PFKM (B), and PDHA1 (C) in juvenile skeletal muscle are shown. Values represent the average of three biological replicates with error bars representing standard error. Wild type = dark bars; I strain = white bars; UEC = unexercised control. Relative expression is given as units of cDNA calculated using a relative standard curve method (see “Materials and Methods”). #,  $p < 0.05$  compared to unexercised control.

## CHAPTER 4

### DISCUSSION

The exercise study demonstrated that of all the exercise groups, adult wild-type mice were most susceptible to training effects. There was a statistically significant increase in the average speed they ran after one week compared to five weeks,  $30 \pm 2$  m/min versus  $48 \pm 5$  m/min respectively. Furthermore, they increased from an average of  $11 \pm 2$  km/day after one week of exercise to  $19 \pm 2$  km/day after five weeks of exercise, illustrating a significant difference ( $p < 0.05$ ). Although similar increases were seen in the juvenile wild-type mice and some of the I strain mice, none were statistically significant, suggesting the adult wild-type mice underwent the most physiological conditioning with the voluntary exercise.

The physiological conditioning observed in the exercise data was not reflected in the heart mass/body mass data. In a study by Allen *et al.* (2001), it was determined that voluntary exercise via wheel-exposure was enough to increase cardiac mass in wild-type C57/Bl6 mice. Such an increase was not observed in the present study comparing exercised mice to their unexercised age-matched controls. After five weeks of exercise, the average heart to body mass ratio did increase slightly in all experimental groups, but not with any

statistical significance. It is possible that given a larger sample size, such as that used in the study by Allen *et al.* (2001) of twelve control mice, the data might reflect these physiological effects more accurately.

Overall the differences in the activity levels of wild-type and the I strain mice are not hard to distinguish, but become statistically significant only after several weeks of acclimation to exercise. The most apparent difference is found in the total time and distance run by the two mice strains, where after five weeks of exercise the I strain mice ran an average of  $211 \pm 23$  km and wild-type mice ran  $418 \pm 4$  km, approximately twice the distance. Although the daily distances and time periods run by the two strains were not significantly different initially, the difference in their activity levels became apparent over the five-week period. This difference in their amount of voluntary activity is surprising when contrasted to the observation made by Lyon *et al.* (1963) that the I strain mice were capable of swimming up to twice as long as wild-type C57/Bl6 mice. One hypothesis may be that the mechanism of metabolism used by the I strain mice as an alternative to glycogen metabolism can sustain increased endurance under forced conditions; however, under voluntary conditions the I strain, while capable of much activity, opt to exercise less than normal mice. Further studies on the methods of metabolism utilized by the I strain mice would have to be conducted.

In Pederson *et al.* (2005), it was suggested that liver glycogen stores may be quantitatively more important in rodent exercise than skeletal muscle glycogen. If we assume that liver glycogenolysis is more important than muscle

glycogenolysis, the results of the exercise study at minimum demonstrate that muscle glycogenolysis is not inconsequential. It is also important to note that the study by Pederson *et al.* (2005) was conducted under forced conditions and the mice were exercised to exhaustion. Under these conditions, it is likely that the abundance of muscle glycogen is insufficient to meet the demand, so hepatic glycogen stores must be used.

Whether or not the I-strain mice would function as a viable mouse model for human glycogenoses is still in question. According to Pederson *et al.* (2005) hepatic glycogen may be more important for exercise than skeletal muscle glycogen, which is not necessarily true of humans. The prevailing view is that skeletal muscle glycogen is indeed a determining factor in human endurance (29, 30). However, liver glycogen stores are known to deplete under strenuous exercise conditions by 50% or more after only one hour (3).

It is also difficult to determine if the clinical symptoms (muscle cramps, weakness, and exhaustion) that are present in human skeletal muscle PhK deficiencies affect the I strain mice and how much stress is needed to induce them. Since it was only after five weeks of voluntary exercise that the I-strain mice showed significant decreases in their activity levels compared to wild-type, one possibility is that the level of intensity of exercise was not significant enough to affect their performance via cramps or exhaustion. As mentioned above, it has also been observed that under forced exercise conditions the I strain mice performed better than wild-type mice (28). However, that does not necessarily

mean that the I strain mice did not experience cramps or muscle pain. In the case of Lyon, *et al.* (1963) the mice were forced to swim to exhaustion, meaning that even if the activity became painful for the mice they may have endured it for the sake of survival.

The present study demonstrates that I strain mice, while capable of voluntary exercise in equitable amounts, show decreased basal activity levels compared to a healthy mouse strain. Initial gene studies did not reveal a significant difference in the expression of key glycolytic and metabolic enzymes between wild-type and I strain mice. Hopefully, further gene studies will be able to identify an alternative energy source or metabolic mechanism that allows the I strain mice to maintain activity for several hours per day without the use of skeletal muscle glycogen. Insight into the intricacies of human glycogenoses will expectedly be gleaned from further study and a deeper understanding of these mice.

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