

**THE INFLUENCE OF CALF SELENIUM STATUS ON GPX-1 AND 3 ACTIVITY AND  
LIVER GPX-1 MRNA**

**A Thesis**

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Genevieve Elizabeth Tanner  
B.S., Louisiana State University  
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## LIST OF ABBREVIATIONS

Se.....	Selenium
GPx.....	Glutathione Peroxidase
ROS.....	Reactive Oxygen Specie(s)
BRD.....	Bovine Respiratory Disease
SeMet.....	Selenomethionine
GPx-1.....	Classical Glutathione Peroxidase
GPx-2.....	Gastrointestinal Glutathione Peroxidase
GPx-3.....	Plasma Glutathione Peroxidase
GPx-4.....	Phospholipid Hydroperoxide Glutathione Peroxidase
GSH.....	Glutathione
H <sub>2</sub> O <sub>2</sub> .....	Hydrogen Peroxide
GSSG.....	Oxidized Glutathione
GSSG-R.....	Glutathione Reductase
NADPH.....	Nicotinamide Adenine Dinucleotide Phosphate
Hb.....	Hemoglobin
EU.....	Enzyme Units
RBC-GPx.....	Erythrocyte Glutathione Peroxidase
UTR.....	Untranslated Region
SECIS.....	Selenocysteine Insertion Sequence
Sec.....	Selenocysteine
tRNA <sup>sec</sup> .....	Selenocysteinyl-tRNA
eEFSec.....	Selenocysteine-Specific Eukaryotic Elongation Factor

SPB2.....	SECIS Binding Protein 2
rpL30.....	Ribosomal protein L30
Ser.....	Serine
t-RNA <sup>[ser]sec</sup> .....	Serine-charged tRNA <sup>sec</sup> Precursor
PCR.....	Polymerase Chain Reaction
Q-PCR.....	Real-Time PCR
BSL.....	Baseline
GFAAS.....	Graphite Furnace Atomic Absorption Spectroscopy
HNO <sub>3</sub> .....	Nitric Acid
Abs <sub>340</sub> .....	Absorption at 340 nm
BSA.....	Bovine Serum Albumin
NaN <sub>3</sub> .....	Sodium Azide
cDNA.....	Complementary DNA
Poly-A.....	Poly-A Polymerase
C <sub>T</sub> .....	Threshold Cycle
ADG.....	Average Daily Gain
ADI.....	Average Daily Intake
GST.....	Glutathione S-transferase

## ABSTRACT

The purpose of this research was to determine the influence of dietary Se on glutathione peroxidase (GPx)-1 and 3 activities and relative liver GPx mRNA levels in growing Holstein bull calves. Calves ( $n = 14$ ) were started 28 d after birth on either a Se adequate (0.15 ppm Se) or deficient (0.01 ppm Se) diet consisting of 3 growth phases and maintained on the diet until 180 d of age. Blood samples were taken from each calf for determination of GPx-1 and GPx-3 activity. Three calves were euthanized at d 21 of age for determination of baseline liver GPx-1 mRNA level. Four calves from each treatment were euthanized at d 180 of age for determination of liver GPx-1 relative mRNA level. Feed intake and average daily gain were not affected by Se level. Mean liver Se concentration was higher ( $P < 0.05$ ) for baseline calves and those fed the Se adequate diet than for calves fed the Se-deficient diet, but there was no difference between baseline calves and Se adequate calves with respect to liver Se concentration. The GPx-1 activity was greater for Se adequate than Se-deficient calves ( $P < 0.01$ ) but not until d 84 of age. The GPx-3 activity was considerably more variable than that of GPx-1 with respect to the trend observed for activity by day, and the GPx-3 activity of the Se-deficient group was only less than that of the Se adequate group ( $P < 0.05$ ) on d 180. N-fold differences were calculated for relative GPx-1 mRNA levels between treatments. There was a 50% decrease in GPx-1 mRNA for Se-deficient calves ( $P < 0.05$ ) compared with the Se adequate calves. Regression analysis also was performed to determine the relationship between the various response variables. There was only a moderate relationship ( $r^2 = 0.58$ ) between GPx-1 mRNA transcript levels and GPx-1 activity at d 180, despite a correlation coefficient of 0.76. The relationship between GPx-1 mRNA transcript level and GPx-3 activity at d 180 was much stronger ( $r^2 = 0.81$ ), with a correlation coefficient of 0.90, which was unexpected, as GPx-3 is generally considered a short-term

indicator of Se status and therefore a much more variable response. Erythrocyte GPx-1 activity was much more sensitive to Se in the diet and thus reflected the diet more closely than did GPx-3. However, GPx-3 activity was more highly correlated to GPx-1 transcript levels. These unexpected results suggest that another trial utilizing larger sample sizes and serial sampling of liver tissue with the sampling of plasma and erythrocytes may provide a clearer picture of the relationship between liver GPx-1 mRNA , tissue Se concentration, and GPx enzyme activities in neonatal and growing Holstein calves.

# CHAPTER I

## INTRODUCTION

Selenium (Se) status is an important indicator of oxidative status and therefore health and disease susceptibility. According to Arthur et al. (2003), “Selenium is essential for the efficient and effective operation of many aspects of the immune system in both animals and humans.” There is increasing evidence concerning nutritional status and immune response in mammals. Although numerous micronutrients, including Fe, Cu, Zn, and Se, have received considerable attention within the last decade (Failla, 2003), it is research regarding Se and immunity that has shown promise to impact health. One of the most surprising findings is that Se deficiency in mice promotes viral mutation and the subsequent development of virulent strains, as demonstrated by the infection of Se-deficient mice with a normally benign virus, Coxsackie virus B3. When Se-deficient mice were infected with Coxsackie virus, there was a significant increase in mortality, while Se-adequate infected mice showed little to no signs of disease, thus indicating an increase in infectivity and virulence as a result of potential genomic mutation in the previously avirulent virus when replicated within a Se-deficient host (Beck et al., 1994a; Beck et al., 1994b; Beck et al., 1994c). The connection between Se status and viral mutagenesis primarily involves oxidative stress (Beck and Levander, 2000) and the role of selenium as an antioxidant is mediated through the Se-dependant glutathione peroxidases (GPx; EC 1.11.1.9) (Rotruck et al., 1973). These are potent antioxidant enzymes responsible for catalysis of the reaction in which reactive oxygen species (ROS) such as hydrogen peroxide are converted to water (Flohe, 1973). Oxidative stress, which results from a build-up of ROS, has been found to have a considerable effect on the evolution of viruses from avirulent strains to virulent strains *in vivo*, an effect that is thought to be due largely to genomic damage incurred by the virus as it is

exposed to a highly oxidative environment (Beck and Levander, 2000). In addition, Se deficiency has been known to have other detrimental effects on the immune system and may also encourage the development of many pro-inflammatory compounds responsible for the predisposition to diseases such as heart disease and cancer (Arthur et al., 2003). Selenium deficiency is also directly linked with the development of white muscle disease and general unthriftiness in cattle and other livestock species (Jenkins and Hidiroglu, 1986).

The potential for Se deficiency to have far reaching negative effects is considerable. An example of this capacity lies with the negative repercussions of oxidative stress in calves, a potentially serious problem within the livestock industry. One of the greatest health impacts of calves during weaning, shipping, and the subsequent feedlot adjustment period is Bovine Respiratory Disease (BRD), a disease comprised of numerous viral, bacteriological, and mycoplasmal components (Ellis, 2001). Bovine Respiratory Disease is the most common and economically detrimental affliction to the beef cattle industry (Snowder et al., 2006) and the disease complex accounts for the majority of feedlot mortality (Loneragan et al., 2001). We hypothesize that selenium deficiency-induced oxidative stress may potentiate not only an increase in the incidence of BRD in unhealthy animals, but also in animals that were previously in good health. Vaccination at weaning with a modified live virus is the most common method for preventing BRD (Snowder et al., 2006) but often little or no concern is given to the selenium status or antioxidative status of these animals at the time of vaccination. Research has shown that protective effects of live-attenuated vaccine often depend on a number of factors, including host nutritional status (Broome et al., 2004), thus the efficacy of such administered vaccines may be in question. Furthermore, *in vivo* studies involving mice (Nelson et al., 2001) have shown that virus replicated in Se-deficient mice results in increased morbidity and mortality when then used

to infect healthy, Se-adequate mice. This has been hypothesized to have serious implications when the co-mingling and transport of calves of unknown health and oxidative status is taken into consideration.

Despite the mounting evidence linking Se deficiency to disease and disease susceptibility, little research is available concerning the Glutathione Peroxidase activity-based categorization of selenium status and deficiency in the postnatal and growing calf. Research has been conducted concerning the effect of Se deficiency in young animals when exposed to agents such as Bovine Rhinotracheitis Virus (Reffett et al., 1988) and Brucellosis vaccine in cattle (Nemec et al., 1990); however, these studies did not attempt to categorize the extent to which these animals were made selenium deficient. Furthermore, little information is available regarding the connection between erythrocyte, plasma, and tissue GPx activity and liver GPx mRNA quantity in selenium deficient versus selenium adequate calves.

The goal of this research is to provide GPx-based categorization of selenium deficiency in postnatal calves to calves at approximately 180 days of age. The hope is that, by providing guidelines for determining the extent of selenium deficiency based on common markers of selenium status, future researchers may continue to further uncover the link between selenium deficiency and disease.

## CHAPTER II

### LITERATURE REVIEW

#### **Selenium in Mammals**

Selenium is considered to be an essential mineral for humans and livestock (Schwarz and Foltz, 1957), its role being primarily in the active site of a number of catalytic enzymes including thioredoxin reductase and the Se-dependant family of glutathione peroxidases. In humans Se deficiency is intimately connected to Keshan disease, which results in cardiomyopathy in affected individuals. Before discovery of the connection of Se deficiency to the disease, Keshan disease was endemic in regions of the People's Republic of China known to be deficient in Se (Gu, 1983; Yang et al., 1988). Selenium deficiency in domestic livestock is expressed primarily as white muscle disease, a debilitating affliction resulting in degeneration and necrosis of both skeletal and cardiac muscle that affects young animals (Underwood, 1981). The resulting lesions occur primarily as a result of oxidative damage to muscle tissue incurred when GPx activity is limited (Gerloff, 1992). In addition, affected animals may exhibit signs of general malaise such as weight loss that may be accompanied by diarrhea (Underwood, 1981), anemia (Morris, et al., 1984), and a decrease in calf weaning weights (Spears et al., 1986). Selenium also has been reported to have implications on reproductive health, as there is considerable documentation regarding the increase of retained placenta incidence in Se-deficient dairy cattle (Harrison and Conrad, 1984; Harrison et al., 1984). Furthermore, abortions, early embryonic death, and infertility are all reproductive complications that have been attributed to Se deficiency (Maas, 1983). Selenium also plays an integral role in the immune system and has been associated with increased intracellular kill of *S. aureus* and *E. coli* by bovine neutrophils (Gyang et al., 1984; Grasso et al., 1990). In cows supplemented with 0.3 ppm Se, intracellular kill of *E. coli* and *S.*

*aureus* was increased in neutrophils, compared with cows that were not supplemented with Se (Hogan et al., 1990). Some studies have even suggested the involvement of Se in both murine (Arthur et al., 2003) and ruminant antibody production and lymphocyte function (Larsen et al., 1988; Reffett et al., 1988). The subclinical effects observed in ruminants involving lymphocyte function and antibody production have been suggested to be mediated via the activity of GPx and, according to Baboir (1984), polymorphonuclear leukocytes are highly sensitive to oxidative damage, due primarily to their use of “oxidative bursts” in intracellular killing.

Selenium absorption occurs primarily in the duodenum with very little absorption by the rumen or abomasum (Wright and Bell, 1966). From the duodenum, Se is carried in the blood to the liver either via carrier proteins or in the free form, where it is then incorporated into selenoproteins, the translation of which is determined genetically (Surai, 2006). While urinary excretion is the primary mode of Se regulation in most non-ruminants, the primary site for excretion of orally administered Se in the ruminant is via the feces. However, when Se is administered either subcutaneously or intravenously, urinary excretion tends to increase versus fecal excretion. In addition, it has been shown that lambs maintained on milk have higher urinary excretion of orally administered Se before rumen development, but that fecal excretion increases as rumen development progresses (Miller and Ramsey, 1988).

The Se requirement for beef cattle is 0.1 ppm, with a maximum tolerable intake of 2.00 ppm (NRC, 2001). Toxicity may occur when extremely high levels of Se are consumed or when moderately high levels are consumed over an extended period of time. Acute Se toxicity primarily occurs most often with the ingestion of plants that have a high affinity for and tend to accumulate Se, but may occur when animals are over-supplemented with Se. When large amounts of these seleniferous plants are consumed, acute toxicity symptoms are usually severe

and include a stifled gait, drooped ears, and lowered head. In addition, animals may exhibit high body temperature, diarrhea, elevated but weak pulse, and labored respiration. If left untreated, acute toxicity may lead to death (Miller and Ramsey, 1988). Chronic toxicity, or alkali disease, can occur when livestock graze cereals, grasses, or hays containing 5 to 50 ppm Se for an extended period of time. Symptoms generally consist of hair loss and sloughing of the hooves. The condition is also characterized by anemia, reduced fertility, stiffness of the joints, and painful hoof lesions (Miller and Ramsey, 1988). While Se toxicity is a concern, Se deficiency is also an important consideration, as previously discussed. Morris et al. (1984), Hidiroglu et al. (1985), and Spears et al. (1986) have reported either clinical or subclinical Se-deficiency in cattle grazing forages containing 0.02 or 0.05 mg/kg Se, although there are reports of calves consuming semi-purified diets containing only approximately 0.02 ppm selenium with no observed clinical signs of deficiency (Reffett et al. 1988; Boyne and Arthur, 1981).

### **Determining Se Status**

Since the discovery by Schwarz and Foltz (1957) that Se was an essential dietary trace element, researchers have been looking for the most accurate and precise method of determining Se status, both in humans and animals. Numerous methods of determining Se status are commonly utilized by laboratories, including determination of serum or plasma Se, whole blood Se, liver and other tissue Se, and erythrocyte and plasma glutathione peroxidase activity (Gerloff, 1992). However, it is these various approaches to assessing Se status that contribute to some confusion in the scientific community concerning exactly what adequate Se status entails. In addition, reports from various labs tend to deviate considerably (Ullrey, 1987), making the task of comparing results between labs difficult. Furthermore, it is somewhat difficult to find research correlating specific experimental results such as tissue, plasma, or whole blood Se concentration

to incidents of Se-deficiency induced diseases like white muscle disease (Gerloff, 1992). As a result, most reports contain data from multiple indicators of Se status and there has been recent focus on correlating such indicators to one another.

### **Se Concentration in Tissues**

One of the most commonly considered assessments of Se status involves concentration of the trace mineral in the body tissues. Tissue Se concentration can be expected to vary with the amount and chemical form of Se in the diet, and in most cases rise with increased Se intake, but not all tissues accrue Se equally (Ullrey, 1987). A study performed by Behne and Wolters (1983) in which rats were fed a commercial diet containing 0.3 ppm Se reported that Se concentrations in tissue, on a wet basis, ranked from highest concentration to lowest in the order of kidney, liver, testes, adrenals, erythrocytes, spleen, pancreas, plasma, lungs, heart, thymus, skeletal muscle, and brain. Studies in cattle of various ages have found that tissue Se concentration declines in order of kidney to liver, heart, and skeletal muscle (Maag and Glen, 1967; Kincaid et al., 1977; Ullrey et al., 1977; Ammerman et al., 1980). In the study reported by Behne and Wolters (1983), Se concentrations in the liver, kidney, erythrocytes, and plasma of rats was 1.29, 1.45, 0.52, and 0.42 ppm Se, respectively (Table 2.1). Assessment of Se status has been performed extensively in various species, including the rat, pig, sheep, chicken, and cow. Whanger and Butler (1988) conducted a trial with male weanling Sprague-Dawley rats in which they fed the rats increasing levels of Se supplemented to a basal diet containing 0.02 ppm. The diets were formulated to contain 0.2, 1.0, 2.0, or 4.0 ppm Se. The researchers found that the rats consuming the basal diet had whole blood, liver, and kidney Se concentrations of  $0.03 \pm 0.01$ ,  $0.02 \pm 0.00$ , and  $0.22 \pm 0.08$   $\mu\text{g Se/g tissue (wet weight)}$ , respectively. Rats consuming diets supplemented with 0.2 ppm Se as sodium selenite had whole blood, liver, and kidney Se

concentrations of  $0.50 \pm 0.06$ ,  $0.72 \pm 0.05$ , and  $1.07 \pm 0.06$   $\mu\text{g Se/g}$  (wet weight), respectively. Rats consuming diets supplemented with 1.0 ppm Se as sodium selenite had whole blood, liver, and kidney Se concentrations of  $0.60 \pm 0.04$ ,  $0.90 \pm 0.10$ , and  $1.53 \pm 0.13$   $\mu\text{g Se/g}$  (wet weight), respectively (Table 2.1). A study was performed by Weiss et al. (1996) in which female rats were fed either a basal diet containing 0.007 ppm Se or a diet supplemented with 0.02, 0.05, 0.1, 0.15, 0.2, or 0.3 ppm Se. The researchers reported that supplementation with 0.02 ppm Se did not significantly increase the liver Se concentration above those rats fed the basal diet and that the liver Se concentration of rats fed the Se-deficient basal diet was 4% the concentration in rats fed the 0.1 ppm diet, on average. Furthermore, liver Se concentration increased sigmoidally with increasing dietary Se.

Swine studies have shown a similar trend regarding Se concentration in body tissues. A study performed by Mahan et al. (1999) was conducted to examine the effect of dietary Se level and source, either organic Se as SeMet or inorganic Se fed as sodium selenite, on tissue Se and various other response variables of grower-finisher pigs. The pigs were bled at trial onset and then at intervals of 30, 60, and 90-d after trial initiation. In the study, the researchers found that swine fed the basal diet containing an average of 0.06 ppm Se had serum Se concentrations of 0.080 ppm at d 0 and then of 0.053, 0.057, and 0.063 on d 30, 60, and 90 of the trial, respectively. Groups of pigs were also supplemented with 0.05, 0.10, 0.20, or 0.30 ppm Se as sodium selenite in addition to the basal diet. No d 0 serum sample was taken for these pigs, but pigs were sampled for serum Se on d 30, 60, and 90 of the trial. On d 30 of the trial, serum Se concentrations for the pigs fed a diet supplemented with 0.05, 0.10, 0.20, or 0.30 ppm Se were 0.098, 0.128, 0.121, and 0.137, respectively. Serum Se concentrations for the same pigs on d 90 of the trial were 0.120, 0.133, 0.139, and 0.152, respectively. When researchers examined liver

**Table 2.1.** Se Concentrations [Se] in Various Tissues of Rats.

<b>Author</b>	<b>Year</b>	<b>[Se] in Diet (ppm)</b>	<b>[Se] in Liver</b>	<b>[Se] in Kidney (ppm)</b>	<b>[Se] in RBC</b>	<b>[Se] in Plasma</b>
Behne and Wolters	1983	0.3	1.29	1.45	0.52	0.42
Whanger and Butler	1988	Basal (0.02)	0.02 ± 0.00	0.22 ± 0.08	0.03±0.01 <sup>a</sup>	-
Whanger and Butler	1988	0.2	0.72 ± 0.05	1.07 ± 0.06	0.50 ± 0.06 <sup>a</sup>	-
Whanger and Butler	1988	1.0	0.9 ± 0.10	1.53 ± 0.13	0.60 ± 0.04 <sup>a</sup>	-

<sup>a</sup> Whole blood.

from a subset of these groups at 55 kg body weight they reported liver Se concentrations of 0.195, 0.425, 0.464, 0.526, and 0.522 ppm for pigs fed either the basal diet or a diet supplemented with 0.05, 0.10, 0.20, or 0.30 ppm Se, respectively. Pigs at 55 kg of body weight were also examined for kidney Se concentrations. When fed either the basal diet containing no added Se or the diets containing 0.05, 0.10, or 0.30 ppm Se, the kidney Se concentrations were 1.305, 1.716, 1.771, and 1.655 ppm Se, respectively. A subset of pigs weighing 105 kg was also killed in order to determine both liver and kidney Se concentration. Pigs fed either the basal diet containing no added Se or the basal diet containing 0.05, 0.10, or 0.30 ppm supplemental Se had liver Se concentrations of 0.258, 0.452, 0.436, and 0.534 ppm Se, respectively. The kidney Se concentrations of the same pigs were 1.811, 2.283, 2.333, 2.328, and 2.376 ppm Se, respectively (Table 2.2). A study performed by Lei et al. (1998) found that plasma Se concentrations for pigs fed a basal diet containing 0.03 ppm Se were 1.25 at d 0 of the trial and 0.18 ppm Se at d 35. Plasma Se concentrations increased in pigs with increasing level of Se supplementation, either the basal and 0.1 ppm Se, or the basal with 0.3 ppm Se added. The plasma Se concentration at d 35 in pigs fed the basal diet supplemented with 0.1 or 0.3 ppm Se were 0.84 and 1.62 ppm Se, respectively. The second experiment reported in the study found that pigs fed a basal diet

containing 0.042 ppm Se had plasma Se concentrations on d 0 of 0.26, 0.24, and 0.23 ppm Se, respectively, while the same pigs at d 35 had plasma Se concentrations of 0.46, 0.49, and 0.58 ppm Se, respectively. The results of both Lei et al. (1998) and Mahan et al. (1999) supported earlier findings by Groce (1973) and Lowry (1985a, b) (Table 2.2).

**Table 2.2.** Se Concentrations [Se] in Various Tissues of Pigs.

Author	Year/Exp	Weight/ Age	[Se] in Diet (ppm)	[Se] in Liver (ppm)	[Se] in Kidney (ppm)	[Se] in Plasma (ppm)
Groce et al.	1971	25 wk	0.15	0.57	2.06	-
Lowry et al.	1985a	8 wk	0.35	0.49	1.36	-
Lowry et al.	1985b	8 wk	0.35	0.50	1.42	-
Lei et al.	1998, Exp 1	9 wk	Basal (0.03)	-	-	0.18 <sup>a</sup>
Lei et al.	1998, Exp 1	9 wk	Basal + 0.1	-	-	0.84 <sup>a</sup>
Lei et al.	1998, Exp 1	9wk	Basal + 0.3	-	-	1.62 <sup>a</sup>
Mahan et al.	1999	55 kg	Basal (0.06)	0.195	1.305	-
Mahan et al.	1999	55 kg	Basal + 0.05	0.425	1.716	-
Mahan et al.	1999	55 kg	Basal + 0.10	0.464	1.771	-
Mahan et al.	1999	55 kg	Basal + 0.30	0.522	1.655	-
Mahan et al.	1999	105 kg	Basal (0.06)	0.258	1.811	-
Mahan et al.	1999	105 kg	Basal + 0.05	0.452	2.283	-
Mahan et al.	1999	105 kg	Basal + 0.10	0.436	2.333	-
Mahan et al.	1999	105 kg	Basal + 0.30	0.534	2.376	-

<sup>a</sup> sampled on d 35 of trial.

Numerous studies have also been performed to assess the effect of Se supplementation on tissue Se concentration in cattle. Maag and Glen (1967) reported that 12 month old steers

consuming 0.11 ppm Se in an unsupplemented diet had kidney and liver Se concentrations of 1.08 to 1.32 ppm and 1.13 to 1.20 ppm, respectively (Table 2.3). Kincaid et al. (1977) found that one month old calves consuming 0.3 ppm Se in an unsupplemented diet had kidney and liver Se concentrations of 2.70 and 0.91 ppm, respectively. In addition, a study performed by Ullrey et al. (1977) in which 15 to 18 month old steers were fed a diet containing approximately 0.18 ppm Se found the animals to have kidney and liver Se concentrations of 1.37 and 0.38, respectively (Table 2.3). A study was also performed by Awadeh et al. (1998) in which growing Holstein heifers were fed either 0.41 or 0.73 ppm Se from  $92 \pm 7$  d of age until approximately 180 d of age. Heifers fed the adequate diet (0.41 ppm Se) had a mean serum Se concentration of 0.115 ppm. Calves fed the excessive diet (0.73 ppm Se) had a serum Se concentration of 0.287 ppm. The same group performed another study in which pregnant, adult crossbred beef cows were fed one of three experimental mineral premixes designed to contain 20, 60, or 120 ppm Se as sodium selenite. The estimated intakes of the three mineral salts were 2.4, 4.7, and 8.7 mg Se/d, respectively. Cows consuming the mineral salt containing 20 ppm Se were found to have a whole blood Se concentration of 0.110 ppm. The same cows were found to have an average serum Se concentration of 0.491 ppm, indicating that over 50% of the Se in the whole blood of those cows was contained in the cellular fraction, most likely in the erythrocytes. Cows consuming mineral premixes containing 60 or 90 ppm had whole blood Se concentrations of 0.154 and 0.192 ppm, respectively, and serum Se concentrations of 0.86 and 0.81 ppm, respectively. Rowntree et al. (2004) reported average plasma selenium concentrations for selenium-drenched pregnant, adult cows that ranged between 0.970 ppm and 0.619 ppm, depending on the time of year. In this study, cows sampled in May had the highest plasma selenium concentrations and when sampled

in September had an average plasma selenium concentration of 0.619 ppm. (SEM=4.61), over 0.30 ppm less than when sampled in May (2004) (Table 2.3).

**Table 2.3.** Se Concentrations [Se] in Various Tissues of Cattle.

Author	Year	Age	[Se] in Diet, ppm	[Se] in Liver, ppm	[Se] in Kidney, ppm	[Se] in Plasma, ppm
Maag and Glen	1967	12 mo old steers	0.11	1.13 to 1.20	1.08 to 1.32	-
Kincaid et al.	1977	1 mo old calves	0.30	0.91	2.70	-
Ullrey et al.	1977	15 – 18 mo old steers	0.18	0.38	1.37	-
Awadeh et al.	1998	~ 180 d old heifers	0.41	-	-	0.115 <sup>a</sup>
Awadeh et al.	1998	~ 180 d old heifers	0.73	-	-	0.287 <sup>a</sup>
Awadeh et al.	1998	Pregnant, adult cows <sup>b</sup>	20 <sup>c</sup>	-	-	0.049 <sup>a</sup>
Awadeh et al.	1998	Pregnant, adult cows <sup>b</sup>	60 <sup>d</sup>	-	-	0.154 <sup>a</sup>
Rowntree et al.	2004	Pregnant, adult cows	20 mg, daily	-	-	0.061 to 0.097
Rowntree et al.	2004	Neonatal heifers <sup>e</sup>	1 mg <sup>f</sup>	-	-	0.072
Rowntree et al.	2004	Neonatal heifers <sup>g</sup>	1 mg <sup>f</sup>	-	-	0.119
Rowntree et al.	2004	Neonatal heifers <sup>h</sup>	1 mg <sup>f</sup>	-	-	0.089

<sup>a</sup> Concentration of Se in serum.

<sup>b</sup> 90 d prepartum to 3 mos postpartum.

<sup>c</sup> Concentration of Se in a supplemental salt. Estimated daily intake per cow was 2.4 mg/d.

<sup>d</sup> Concentration of Se in a supplemental salt. Estimated daily intake per cow was 4.7 mg/d.

<sup>e</sup> d 0 of age.

<sup>f</sup> one-time injection, immediately after birth.

<sup>g</sup> d 1 of age.

<sup>h</sup> d 6 of age.

Often of interest is the effect of cow Se status on Se concentrations in the tissues of neonatal and growing calves, because it has been shown that Se effectively passes through the placental barrier in cattle (Van Saun et al., 1989). Early signs of Se-deficiency as well as other

mineral deficiencies in ruminants are often expressed in the young of deficient animals as, in the case of Se, white muscle disease or neonatal ataxia (Abdelrahman and Kincaid, 1993).

Therefore, tissue reserves of such nutrients in the fetal and neonatal calf are of considerable interest. Abdelrahman and Kincaid (1993) measured liver Se concentration in fetuses of various ages removed from cows at slaughter and found that, on average, Se concentration increased from d 145 to d 195, but then decreased from d 195 to d 245. The researchers concluded that this decrease in liver Se concentration during late gestation might possibly reflect an increase in the synthesis of selenoproteins such as GPx by the fetus prepartum, and therefore an increased need for Se by the fetus. A study performed by Weiss et al. (1984) found that Holstein calves from cows fed 0, 1, or 5 mg of supplemental Se as sodium selenite 60 d before parturition had serum Se concentrations that mirrored that of their respective dams. When the serum Se concentrations of calves at birth were regressed against serum Se concentrations of their dams at 60 d prepartum, or d 0 of the trial, there was virtually no correlation ( $r^2 = 0.01$ ). However, when the serum Se concentrations of the same calves were then regressed on the serum Se concentrations of their dams at 45, 30, 15, and 0 d prepartum, there was significant correlation ( $r^2 = 0.79, 0.79, 0.83, \text{ and } 0.73$ , respectively). Furthermore, calves whose dams received 1 mg supplemental Se maintained higher serum Se concentration longer than those calves whose dams received no supplemental Se. The researchers interpreted this data to suggest that Se concentration in the blood of prepartum cows can be used to predict neonatal calf Se status and that supplementation of the dam may be a method of increasing calf Se concentrations before parturition. In addition, cow Se status may also continue to play a significant role in the Se status of their calves after parturition, primarily if calves continue to suckle cows, as occurs in beef cattle, since Se is also transferred through the milk of lactating females (Rowntree et al., 2004; Bruzelius et al., 2007).

Ammerman et al. (1980) reported that kidney and liver Se concentrations of calves suckling cows maintained on a diet consisting of approximately 0.2 ppm Se (0.1 ppm occurring naturally and 0.1 ppm supplemented Se) and having an average liver Se concentration of 0.2 ppm were 1.22 and 0.11 ppm, respectively.

Given the wide range of Se concentrations and the considerable amount of variability between different laboratories, some researchers have attempted to provide ranges of Se concentrations for use as a Se status guideline. In the review titled “Biochemical and Physiological Indicators of Selenium Status in Animals” (1987), Ullrey states that plasma Se concentrations of 0.08 to 0.12 ppm may be considered adequate. The Michigan State University Animal Health Diagnostic Laboratory has also attempted to provide such ranges for reference. According to their laboratory, normal plasma selenium concentrations for adult cattle are expected to range from 0.070 to 0.100 ppm and serum selenium values generally increase gradually as animals age, with initial serum selenium values of approximately 0.050 to 0.080 ppm for neonates considered normal (Stowe and Herdt, 1992). The Michigan State University Animal Health Diagnostic Laboratory analyzed serum samples for cattle of various ages and found that, on average, young calves aged 10 to 29 days of age had serum selenium concentrations between 0.055 and 0.075 ppm, and between days 30 and 180 of age, calves typically had serum selenium concentrations between 0.060 and 0.080 ppm (Stowe and Herdt, 1992). According to Gerloff (1992), marginally Se-deficient adult cattle generally have plasma selenium concentrations between 0.040 and 0.070 ppm, while adult cattle with concentrations below 0.040 ppm are considered deficient. Liver selenium concentrations generally range between 1.2 and 2.0 ppm on a dry weight basis and are typically consistent in normal animals of adequate selenium status, regardless of animal age (Stowe and Herdt, 1992).

Although plasma, whole blood, and tissue selenium concentrations are commonly measured, these response variables are not generally recognized as being accurate indicators of selenium status (Gerloff, 1992). This is primarily due to the fact that Se may be absorbed in one of two forms, inorganic, usually as sodium selenite, or in the organic form, most commonly as selenomethionine (SeMet), which is incorporated nonspecifically into protein as an amino acid in which the sulfur residue has been replaced by Se. Selenomethionine is not incorporated into Se-dependent GPx, considered to be one of the most common functional forms of Se, thus assay for Se in whole blood, serum, plasma, or other tissues alone may not provide an accurate depiction of functional Se status (Burk, 1991). In fact, an increase in tissue Se concentration is not always associated with a corresponding increase in GPx activity (Lane et al., 1979; Elsey et al., 1983; Whanger and Butler, 1988). In addition, when serum Se concentration was regressed via simple linear regression on whole blood Se concentration, the correlation coefficients were moderately high ( $r^2=0.62$  and  $0.88$  for HG-AAS data and ICP data, respectively). However, prediction intervals calculated from these correlation coefficients for estimating whole blood Se from serum Se were very wide (Maas, 1992). For this reason, most conclude that serum Se concentration is not necessarily an appropriate indicator of nutritional Se status. The researchers theorized that these wide intervals were possibly due to short-term changes in serum Se concentration due to variable Se intake short-term, whereas whole blood, or erythrocyte, Se concentration reflects more long-term Se status (Maas, 1992). Given these drawbacks to the sole use of Se concentration as an indicator of Se status, GPx activity is generally considered a more accurate indicator of Se status, but not all forms of the enzyme serve equally in this role.

## **The Se-Dependent Glutathione Peroxidases**

The role of Se in the body is primarily as an antioxidant via the Se-dependent glutathione peroxidases (Arthur et al., 2003). Using erythrocytes extracted from rats maintained on either a Se-adequate or Se-deficient diet, Rotruck et al. (1973) found that hemoglobin oxidation was increased in the erythrocytes of rats maintained on the Se-deficient diet. Further investigation by the group showed that Se was a “necessary and integral” part of the GPx enzyme. In addition, Flohe et al. (1973) showed that the GPx enzymes contained 4 g atoms of Se mol<sup>-1</sup>. Since the discovery of Se in the active site of GPx by Rotruck et al. (1973) and Flohe et al. (1973), four primary Se-dependent GPx enzymes have been identified: Classical Glutathione Peroxidase (GPx-1; EC 1.11.1.9), Gastrointestinal Glutathione Peroxidase (GPx-2; 1.11.1.9), Plasma Glutathione Peroxidase (GPx-3; 1.11.1.9), and Phospholipid Hydroperoxide Glutathione Peroxidase (GPx-4; EC 1.11.1.2). Each of the Se-dependent GPx enzymes, with the exception of GPx-4 (Lei et al., 1995), is a homotetramer (Takahashi et al., 1987) containing one selenocysteine per subunit in the active site of the enzyme (Forstrom et al., 1978) and each subunit has a molecular mass of approximately 19 kD (Epp et al., 1983). The primary function of the GPx enzyme is as an antioxidant responsible for catalysis of the reaction in which glutathione (GSH) in the reduced form is used to reduce cellular reactive oxygen species (ROS), primarily hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), common byproducts of cellular metabolism and processes involving oxygen (Flohe, 1973). These strong oxidants have the potential to react with cellular macromolecules and cause damage such as genomic mutations, destruction of protein structure and function, and lipid peroxidation, which may, in turn, result in considerable cellular oxidative stress and even cell death (Halliwell et al., 1992; Poot, 1991). In order to avoid such damage, these ROS are converted to water (H<sub>2</sub>O) or, in the case of organic hydroperoxides, non-toxic

alcohols. Nicotinamide Adenine Dinucleotide Phosphate (NADPH) then serves as a hydrogen donor to the oxidized GSH, returning it to the active reduced form. Specifically, two glutathione molecules are oxidized to form two molecules of water from one molecule of H<sub>2</sub>O<sub>2</sub> via the catalytic enzyme GPx. The oxidized glutathione (GSSG) is then reduced via catalysis by the enzyme glutathione reductase (GSSG-R) to reform two molecules of glutathione (Paglia and Valentine, 1967) (Figure 2.1).



**Figure 2.1.** The oxidation and subsequent reduction of GSH, from Paglia and Valentine (1967).

### **Glutathione Peroxidase-1 (GPx-1)**

Since the discovery (Mills, 1957) and partial purification (Mills, 1959) of GPx, numerous forms of the enzyme have been isolated and purified in various body tissues. The form of GPx contained within the cell cytosol and mitochondria is GPx-1, or cytosolic GPx (Chambers et al., 1986; Esworthy et al., 1997). The highest activity of this enzyme is generally considered to be in the liver, followed by the erythrocytes, lungs, and heart (Ullrey, 1987). Considerable research has shown that GPx-1 functions primarily in an anti-oxidant role, its primary substrate being H<sub>2</sub>O<sub>2</sub> and other organic hydroperoxides. Using GPx knockout mice possessing a homozygous null mutation for GPx-1, Haan et al. (1998) demonstrated that the enzyme has a significant role in the protection of cells from paraquat-induced oxidation. The GPx-1 knockout mice were shown to have significantly increased mortality when exposed to 30 ppm paraquat, with 100% of knockouts dying within 5 hours of exposure, versus the controls, which showed no evidence of toxicity. The group also showed that cortical neurons, when challenged directly with H<sub>2</sub>O<sub>2</sub>, were considerably more susceptible to oxidative damage in the knockout mice than in the control group. Furthermore, Haan et al. (1998) showed that exposure to paraquat transcriptionally up-

regulated GPx-1 in normal cells, reinforcing the importance of the enzyme in protection from oxidative stress. Recent studies utilizing GPx-1 knockout mice also have shown that these animals are actually healthy and fertile up to 15 month of age, showing no considerable growth response which may be attributed to Se-deficiency or, as a result, GPx-1 deficiency (Ho et al., 1997; Cheng et al., 1997). These findings agree with other observations using Se-deficiency to induce a decrease in GPx activity in various species (Christiansen and Burgener, 1992; Lei et al., 1995; Mahan and Parrett, 1996; Weiss et al., 1996; Lei et al., 1998; Haan et al., 1998; Lawler et al., 2004; Payne and Southern, 2005). These reports are in conflict with findings of Whanger and Butler (1988) in which rats fed a basal diet containing 0.02 ppm Se gained significantly less than those rats fed a diet supplemented with varying levels of Se. However, the researchers reported that the rats consuming the basal diet consumed significantly less feed than those rats fed the basal diet supplemented with varying levels of Se.

Glutathione Peroxidase activity in blood is associated largely with erythrocytes, which contain approximately 98% of total GPx-1 activity (Scholtz and Hutchinson, 1979). The enzyme is incorporated into reticulocytes, the precursors of erythrocytes, during their formation with whole-body erythrocyte turnover only occurring approximately every 90 to 120 days in the bovine (Ullrey, 1987), marking erythrocyte GPx-1 (RBC-GPx) activity as an ideal estimator of long-term Se status. However, there are notable inter-species variations with regard to GPx activity in erythrocytes. A comparison of GPx activity in nine species was published by Suzuki et al. (1985). The means of the nine species ranged from 22 EU/g hemoglobin (Hb) in the golden hamster to 493 EU/g Hb in the mouse, where one enzyme unit (EU) is equal to the amount of GPx activity required to oxidize 1  $\mu$ mol NADPH per min. The GPx activity in the erythrocytes of cattle was reported to be  $165 \pm 43$  EU/g Hb (Suzuki et al., 1985) (Table 2.4). A study

performed by Whanger and Butler (1988) found that rats fed a basal diet containing 0.02 ppm Se had an average RBC-GPx activity of  $47 \pm 11$  EU/g Hb. When rats were fed the basal diet supplemented with 0.2 ppm Se or 1.0 ppm Se as sodium selenite, they had an average RBC-GPx activity of  $435 \pm 19$  EU/g Hb and  $518 \pm 21$  EU/g Hb, respectively (Table 2.5). Weiss et al. (1996) found that RBC-GPx activity in Se-deficient rats maintained on a diet containing 0.007 ppm Se was  $40 \pm 3\%$  of the activity in Se adequate female rats fed a diet containing 0.1 ppm Se as sodium selenite. Erythrocyte GPx-1 activity ranged from approximately 80 EU/g Hb for rats consuming the Se-deficient basal diet containing 0.007 ppm Se to approximately 280 EU/g Hb for those rats fed a diet supplemented with 0.3 ppm Se as sodium selenite (Table 2.5).

Erythrocyte GPx-1 activity has also been used as an indicator of Se status in cattle at various production stages. In a study examining the effect of pre- and post-partum Se supplementation on cows and their calves, cows consuming 13 mg Se/d for 15 d pre-partum had an average d 0 RBC-GPx activity of 2.33 EU/g Hb. When sampled at 15 and approximately 54 d post-partum, the same cows had an average RBC-GPx activity of 122.4 EU/g Hb and 127.2 EU/g Hb at respectively. Calves from these cows had RBC-GPx activity of 186.2 and 129.3 EU/g Hb at 15 d and 54 d post-partum, respectively. In the same study, cows consuming 32.5 mg Se/d had an initial RBC-GPx activity of 1.67 EU/g Hb. When these cows were sampled at d 15 and 54 post-partum, they had an average RBC-GPx activity of 161.7 and 181.1 EU/g Hb, respectively (Enjalbert et al., 1999). A second experiment conducted by the same group found that when cows were supplemented with 0, 13, or 32.5 mg Se/d for 15 d postpartum and sampled approximately 101 d post-partum, they had an average RBC-GPx activity of 67.2, 146.4, and 150.9 EU/g Hb, respectively (Table 2.5). The calves of the same cows were treated with 1.38 mg Se intramuscularly on d 2 post-partum and again at approximately 49 d of age. When these

calves were sampled at approximately 101 d post-partum, they had an average RBC-GPx activity of 130.0, 156.4, and 177.1 EU/g Hb when their dams received 0, 13, or 32.5 mg Se/d, respectively (Enjalbert, 1999). In a study conducted by Rowntree et al. in 2004 involving

**Table 2.4.** Average RBC-GPx-1 Activity of Animals and Humans, from Suzuki et al., 1985.

<b>Species</b>	<b>EU/g Hb<sup>a,b</sup></b>
Golden hamster	22 ± 2
Guinea Pig	28 ± 6
Rabbit	33 ± 7
Rat	106 ± 20
Mouse	493 ± 124
Goat	115 ± 53
Sheep	124 ± 41
Cattle	165 ± 43
Human	35 ± 7

<sup>a</sup> Mean ± 95% confidence limits.

<sup>b</sup> EU= Enzyme Unit; 1 EU is the amount GPx activity required to oxidize 1 µmol NADPH per min.

multiparous Hereford cows, erythrocyte GPx activities in cows unsupplemented with Se were 14.94 EU/g Hb, while RBC-GPx activity in cows supplemented with Se was 24.61 EU/g Hb (SEM=3.33).

Glutathione Peroxidase-1 is also found in animal tissues, most notably the liver, lungs, and heart (Ullrey, 1987). It is often desirable to examine GPx-1 activity in tissues in an attempt to both accurately determine Se status and to correlate tissue activity to tissue Se concentrations and GPx-1 mRNA concentrations. Liver is often used for this purpose as it has been shown to have the highest GPx activity of any tissue that has been analyzed (Behne and Wolters, 1983).

Knowles et al. (1999) reported that liver GPx-1 activity was positively correlated to both liver Se concentration ( $r^2= 0.73$ ) and, to a lesser extent, blood Se concentration ( $r^2= 0.69$ ). Liver GPx-1 activity is also highly responsive to Se status. Liver GPx activity in Se-deficient rats has been

**Table 2.5.** Erythrocyte GPx-1 activity in various species fed varying levels of Se.

Author	Year	Species	[Se] in diet, ppm	RBC-GPx-1, EU/g Hb <sup>a</sup>
Whanger and Butler	1988	Rat	0.02	47 ± 11
Whanger and Butler	1988	Rat	0.2	435 ± 19
Whanger and Butler	1988	Rat	1.0	518 ± 21
Weiss et al.	1996	Rat	0.007	80
Weiss et al.	1996	Rat	0.3	280
Enjalbert et al.	1999	Cow	10 <sup>b,c</sup>	67.2 <sup>d</sup>
Enjalbert et al.	1999	Cow	13 <sup>b</sup>	146.4 <sup>d</sup>
Enjalbert et al.	1999	Cow	32.5 <sup>b</sup>	150.9 <sup>d</sup>
Rowntree et al.	2004	Cow	No supplemental	14.94
Rowntree et al.	2004	Cow	20 mg drench	24.61

<sup>a</sup> EU= Enzyme Unit; 1 EU is the amount GPx activity required to oxidize 1 μmol NADPH per min.

<sup>b</sup> mg Se d<sup>-1</sup>.

<sup>c</sup> supplemented for 15 d postpartum.

<sup>d</sup> at 101 d post-partum.

previously reported to be as little as 8% of that observed in rats maintained on a Se-adequate diet formulated to contain 0.5 ppm Se (Lawrence and Burk, 1976). Whanger and Butler (1988) found that rats maintained on a Se-deficient basal diet containing 0.02 ppm Se had an average liver GPx activity of 8 ± 4 EU/g protein, while rats fed the basal diet supplemented with 0.2 ppm Se as sodium selenite had an average liver GPx activity of 700 ± 105 EU/g protein (Table 2.6).

Weiss et al. (1996) reported average liver GPx-1 activity of rats consuming a basal diet

containing 0.007 ppm Se to be less than 100 EU/g protein, only 2% of the activity observed in rats fed a diet containing 0.1 ppm Se as sodium selenite. In this study, the liver GPx activity of the supplemented rats was, on average, approximately 2,400 EU/g protein (Table 2.6). Liver GPx-1 activity levels have also been determined in swine; growing pigs maintained on a Se-deficient basal diet containing 0.03 ppm Se were found to have an average liver GPx-1 activity of approximately 12.5 EU/g protein, while pigs fed the basal diet supplemented with either 0.1 or 0.3 ppm Se as sodium selenite had average liver GPx-1 activities of 50 and 210 EU/g protein, respectively (Lei et al., 1998, Table 2.6).

**Table 2.6.** Liver GPx-1 activity in various species fed varying levels of Se.

<b>Author</b>	<b>Year</b>	<b>Species</b>	<b>[Se] in diet, ppm</b>	<b>Liver GPx-1, EU/g protein</b>
Whanger and Butler	1988	Rat	0.02	8 ± 4
Whanger and Butler	1988	Rat	0.2	700 ± 105
Weiss et al.	1996	Rat	0.007	< 100
Weiss et al.	1996	Rat	0.1	2,400
Lei et al.	1998	Pig	0.03	12.5
Lei et al.	1998	Pig	0.1	50
Lei et al.	1998	Pig	0.3	210

### **Glutathione Peroxidase-3 (GPx-3)**

In comparison to that of erythrocytes, plasma Se levels are more sensitive to short-term changes in Se supplementation and tend to fluxuate depending on Se concentration, frequency of Se intake, and even hydration status. Thus, extracellular plasma GPx-3 activity is considered to reflect short-term Se status and is considered inappropriate as a long-term indicator of Se status

(Gerloff, 1992). Since its discovery in 1986 (Takahashi and Cohen), research has shown that GPx-3 is actually regulated separately from GPx-1, illustrated in part by research showing that Se repletion results in an increase in GPx activity at different times for erythrocytes versus plasma. Plasma GPx activity increases relatively quickly in comparison to the rate of increase of RBC-GPx activity, for which there is a significant lag-time of approximately four to five weeks in humans. This lag is due to the fact that GPx-1 may only be incorporated into the erythrocyte during erythropoiesis (Cohen et al., 1985). An additional experiment in which rats both genetically altered to overexpress GPx-1 and GPx-1-normal rats were fed either a diet adequate or deficient in Se further supports the work of Cohen et al. (1985). In this experiment, the overexpression of GPx-1 failed to have any bearing on the expression of GPx-3 (Cheng et al., 1997). In addition, when GPx-1 knockout mice were fed one of two diets containing either 0.002 ppm Se or 0.5 ppm supplemented Se as sodium selenite, researchers observed no effect of the knockout of the GPx-1 gene on GPx-3 activity or GPx-3 mRNA expression in the kidney (Cheng et al., 1997). Whereas the primary site of GPx-1 synthesis is the liver, the primary site of GPx-3 mRNA and protein synthesis is the proximal tubules of the kidney (Avissar et al., 1994). Furthermore, sequencing of the purified protein (Esworthy et al., 1991) and its cDNA (Takahashi et al., 1990) have demonstrated that GPx-3 is a separate gene product from that of GPx-1, in spite of previous belief that the two resulted from post-transcriptional modification of the same gene.

As with tissue Se concentration and GPx-1 activity, there is considerable inter-species and inter-lab variation with respect to reported activity (Ullrey, 1987). Lei et al. (1995) found that rats fed a basal diet containing 0.002 ppm Se had an average GPx-3 activity of  $2.00 \pm 0.58$  EU/g protein, while rats fed a diet supplemented with 0.130 ppm Se as sodium selenite had an

average GPx-3 activity of  $95.33 \pm 14.15$  EU/g protein (Table 2.7). Weiss et al. (1996) found that plasma GPx-3 activity in rats fed a Se-deficient basal diet was  $8 \pm 1\%$  that of rats fed a diet supplemented with 0.1 ppm Se as sodium selenite. Plasma GPx-3 activity for the deficient rats was approximately 1.0 EU/g protein while activity for rats maintained on a diet supplemented with 0.1 ppm of diet was approximately 800.0 EU/g protein (Table 2.7). Research with swine has

**Table 2.7.** GPx-3 activity in various species fed varying levels of Se.

Author	Year	Species	[Se] in diet, ppm	GPx-3 activity, EU/g protein <sup>a</sup>
Lei et al.	1995	Rat	0.002	$2 \pm 0.58$
Lei et al.	1995	Rat	0.130	$95.33 \pm 14.15$
Weiss et al.	1996	Rat	0.007	1.0
Weiss et al.	1996	Rat	0.1	800.0
Lei et al.	1998	Pig	0.03	< 1.0
Lei et al.	1998	Pig	0.1	> 10.0
Lei et al.	1998	Pig	0.3	>15.0
Southern and Payne	2005	Chicken (Broiler)	0.067	$< 0.1 \times 10^5 - 0.05 \times 10^5$
Southern and Payne	2005	Chicken (Broiler)	0.3	$3 \times 10^5 - 3.5 \times 10^5$
Rowntree et al.	2004	Multiparous cow	No supplemental	0.92
Rowntree et al.	2004	Multiparous cow	20 mg drench	1.34

<sup>a</sup> EU= Enzyme Unit; 1 EU is the amount GPx activity required to oxidize 1  $\mu$ mol NADPH per min.

shown that pigs fed a basal diet containing 0.03 ppm Se had an average GPx-3 activity of less than 1.0 EU/g protein after 35 d on the diet (Lei et al., 1998). Pigs fed the basal diet supplemented with either 0.1 ppm Se or 0.3 ppm Se as sodium selenite had an average GPx-3 activity of greater than 10 EU/g protein and greater than 15 EU/g protein, respectively, after 35 days on their respective diets (Lei et al., 1998, Table 2.7). Southern and Payne (2005) performed

a study examining the effect of Se source in broilers. After being fed either a Se-deficient basal corn-soybean meal diet containing 0.067 ppm Se with no supplemented Se or the basal diet supplemented with 0.3 ppm Se as sodium selenite for 10 d, broilers had an average GPx-3 activity of less than  $0.1 \times 10^5$  EU/g protein and between 3 and  $3.5 \times 10^5$  EU/g protein, respectively (Table 2.7). In addition, when the broilers initially fed the Se supplemented diet were then fed the Se-deficient basal diet for an additional 12 d, GPx-3 activity fell to between 0.1 and  $0.05 \times 10^5$  EU/g protein. GPx-3 activity of the broilers maintained on the Se-deficient basal diet for the full 22 d period decreased only slightly further to  $0.05 \times 10^5$  EU/g protein. In a study conducted by Rowntree et al. in 2004 involving multiparous Hereford cows, plasma GPx-3 activities were reported to be 0.92 EU/g protein in unsupplemented cows versus 1.34 EU/g protein in supplemented cows (Table 2.7).

### **Selenium Regulation of GPx**

Selenium is found in the active site of all Se-dependent GPx enzymes, thus marking the element as a significant regulator of Se-dependent enzyme activity, especially the activity of GPx-1 (Sunde, 1997). The decrease in enzyme activity during Se-deficiency is due to a decrease in the level of the GPx enzyme protein (Knight and Sunde 1987; Knight and Sunde, 1988). Most studies examine the effect of Se-deficiency on GPx-1 activity, as it is the most dramatically affected GPx enzyme (Weiss and Sunde, 1998). This is primarily because GPx-1 appears to compete for Se less efficiently than other selenoproteins in nutritional Se-deficiency (Lei et al., 1995). In addition to a loss in enzyme activity, a significant decrease in GPx mRNA is also observed in Se-deficiency, resulting in a reduction of liver GPx-1 mRNA levels to less than 10% of those levels observed in Se-adequate rats (Saedi et al., 1988; Yoshimura et al., 1988; Christensen and Burgener, 1992; Hill et al., 1992). Although all of the Se-dependent GPx

enzymes are regulated by the availability of Se, some are affected to a greater extent than others (Moriarty et al., 1998). When comparing the Se regulation of GPx-1 mRNA to other selenoprotein mRNAs, most researchers have found that GPx-1 mRNA levels decrease considerably in Se-deficiency, whereas the levels for most other selenoproteins are affected less dramatically (Hill et al., 1992; Lei et al., 1995; Weiss et al., 1997). In fact, GPx-4 mRNA levels are not significantly reduced in Se-deficiency (Lei et al., 1995), although GPx-1 and 4 share approximately 40% homology in their nucleotide sequences (Sunde et al., 1993). This down-regulation has been further shown to be specific for GPx-1 mRNA using Se-repletion studies in which rats were first maintained on a Se-deficient diet and were then fed a diet adequate in Se. While internal control genes such as Poly-A polymerase mRNA, 18S rRNA, and  $\beta$ -actin mRNA levels remained unaffected by Se-deficiency, GPx-1 mRNA levels dropped dramatically (Lei et al., 1995; Bermano et al., 1996; Weiss et al., 1996; Weiss et al., 1997). When the rats were then fed a diet adequate in Se, GPx-1 activity and mRNA levels increased rapidly. However, in spite of the rapid decrease observed in GPx-1 activity and level during Se depletion followed by the rapid increase observed in both during Se repletion, GPx-1 transcription levels are not affected by deficiency (Toyoda et al., 1990; Christensen and Burgener, 1992). Furthermore, transcription of the Se-dependent GPx genes by cultured cells are minimally effected by the availability of Se in the culture medium (Chada et al., 1989) and the transport of GPx mRNA out of the nucleus of the cell has also been shown to be unaffected by Se status (Sugimoto and Sunde, 1992). In addition, studies utilizing isolated rat liver cells showed that Se-deficiency reduced the relative abundance of cytoplasmic GPx-1 mRNA but did not have any effect on nuclear GPx-1 mRNA (Moriarty et al., 1998). These results have led researchers to conclude that the regulation of GPx by Se is via a posttranscriptional mechanism (Sunde, 1994) and that induced Se deficiency

regulates the abundance of cytoplasmic GPx-1 mRNA, most likely by eliciting the decay of cytoplasmic mRNA via nonsense-codon-mediated decay (Moriarty et al., 1998).

### **The SECIS Element and UGA-mediated Nonsense Decay**

All eukaryotic selenoprotein mRNAs contain a *cis*-acting element in the 3' untranslated region (UTR) that is required for the insertion of Se (Kinzy et al., 2005). This sequence is known as the selenocysteine insertion sequence, or SECIS element, which consists of an RNA hairpin loop composed of two helices separated by an internal loop (Walczak et al., 1996; Martin et al., 1998). This loop contains two highly conserved sequences: four non-Watson and Crick base pairs consisting of UGAU and AAA in the stem and loop, respectively (Shen et al., 1993). The four consecutive non-Watson and Crick base pairs located within the core of helix 2 are responsible for conveying a structural/functional motif that is necessary for selenocysteine (Sec) insertion during translation (Walczak et al., 1996; Walczak et al., 1998). In addition, a UGA codon, which normally signals translational termination, is located within the open reading frame and, in combination with the SECIS element located downstream within the 3' UTR, codes for the insertion of Sec, the 21<sup>st</sup> amino acid and the form in which Se is incorporated into selenoproteins (Berry et al., 1991; Berry et al., 1993; Low and Berry, 1996). The recognition of this codon as an insertion sequence for Sec requires the presence of Se and a metabolic pathway that converts Se to selenocysteinyl-tRNA (tRNA<sup>sec</sup>) (Moriarty et al., 1998). Research has shown that adding the GPx stem-loop structure to mRNA encoding a protein normally lacking a Se moiety resulted in the inclusion of Sec in the protein and subsequent Se regulation. The researchers interpreted this as an indication that the GPx 3'-UTR alone is adequate for signaling the translation of a UTR as Sec (Shen et al., 1993). However, further work has shown that Sec incorporation also requires three additional *trans*-acting elements, a Sec-specific translation-

elongation factor (eEFSec), SECIS binding protein 2 (SBP2), and ribosomal protein L30 (rpL30) (Kinzy et al., 2005).

In addition to contributing to the incorporation of Se into selenoproteins, the combined function of the UGA codon and the SECIS element are considered to be responsible for mediating the nonsense decay of cytoplasmic GPx mRNA in the absence of adequate Se (Moriarty, 1998). The modification of cytosolic mRNA turnover by a nutrient for the regulation of eukaryotic gene expression is not an unusual concept. In numerous cases, the availability of nutrients serves to alter gene expression for products that are relevant to their metabolism (Christensen and Burgener, 1992). Iron availability, for example, can alter the expression of transferrin by the binding of iron to the cytosolic transferrin mRNA receptor, which in turn stabilizes the mRNA, protecting it from degradation (Klausner and Harford, 1989; Müllner et al., 1989). In addition, there are numerous organisms including various species of bacteria, yeasts, and vertebrates that have mechanisms for the elimination of mRNAs that have prematurely terminated because of frameshift or nonsense mutations (Peltz et al., 1994; Maquat, 1995; Ruiz-Echevarria, 1996). Interestingly, the majority of mammalian cells that have been studied have mRNAs that are degraded while associated with the nucleus (Maquat, 2005); however, selenoprotein mRNAs decay after transport into the cytoplasm, underlined by the fact that Se-deficiency results in a decrease in cytoplasmic GPx-1 mRNA but not in a decrease in nuclear GPx-1 pre-mRNA or mRNA (Moriarty, 1998). The combined work of Weiss and Sunde (1997) and Moriarty (1998) suggest that the combination of the UGA codon and the SECIS element are required for the decrease in GPx-1 mRNA abundance as a result of Se-depletion. Kinzy et al. (2005) reinforced this model of UGA codon nonsense-mediated decay while characterizing the SBP2 complex. According to his group, during translation, when the ribosome pauses at the

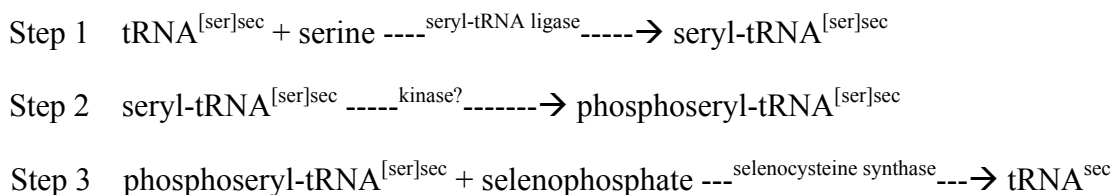
UGA codon, the SECIS element is recognized by SPB2, which is bound to the ribosome. As SBP2 binds to the SECIS element, it briefly releases the ribosome, allowing access to the ribosomal A-site and incorporation of the tRNA<sup>sec</sup> via the eEFSec/tRNA<sup>sec</sup> complex. In the absence of Se, there is no t-RNA<sup>sec</sup> to add Sec to the growing protein and translation is thus terminated. It is currently unknown whether the nonsense-mediated decay of the mRNA that follows occurs while the truncated protein and mRNA are still associated with the ribosome or if it occurs within “cytoplasmic foci”, ribosome-free sites of general mRNA decay (Maquat, 2005).

### **Incorporation of Se into GPx and Other Selenoproteins**

Selenocysteine is located 41 residues from the N-terminal end of rat liver GPx (Condell and Tappel, 1982), 45 residues from the end of bovine GPx (Gunzler et al., 1984), and 47 residues from the end of murine GPx (Chambers et al., 1986). In 1987 Sunde and Evenson elucidated the origin of the Sec residue by perfusing isolated rat liver with amino acids labeled with either C<sup>14</sup> or H<sup>3</sup>. They determined that the skeletal precursor to Sec was not cysteine, as previously thought, but serine (Ser).

The formation of Sec outlined by Burk (1991) is based primarily on the mechanism as is understood in *E. coli* and requires both a Ser-charged tRNA and a source of selenide. However, there are indications that a similar scheme exists in mammals (Burk, 1991; Hubert et al., 1996; Kinzy et al., 2005) This mechanism requires a unique tRNA (tRNA<sup>[ser]sec</sup>) that is first charged with a Ser residue by the catalytic enzyme seryl-tRNA synthetase, as Sec synthesis occurs on its tRNA (Driscoll and Copeland, 2003). The Ser must then be activated to phosphoseryl-tRNA<sup>[ser]sec</sup> before it can be replaced by Se. It is thought that this step is carried out by a kinase in animals, but the specific mechanism is not currently known (Lee et al., 1989). The conversion of

phosphoseryl-tRNA<sup>[ser]sec</sup> to tRNA<sup>sec</sup> with selenophosphate as the Se donor is then catalyzed by selenocysteine synthase (Hubert et al., 1996) (Figure 2.2).



**Figure 2.2.** Eukaryotic selenocysteine synthesis, from Burk (1991), with changes.

The addition of Sec to selenoproteins, including GPx, is a co-translational event in which the UGA codon codes for the addition of Sec via tRNA<sup>sec</sup>. As previously outlined, during translation of the selenoprotein mRNA, SBP2 is bound to the translating ribosome. The elongation factor eEFsec serves to escort tRNA<sup>sec</sup> to the ribosomal A-site (Fletcher et al., 2001). When the ribosome arrives at the UGA codon, it pauses, allowing SBP2 to recognize both specific nucleotides within the SECIS element and the eEFSec/tRNA<sup>sec</sup> complex. SECIS binding protein 2 then binds both the SECIS element and eEFSec and is temporarily released from the ribosome, allowing the eEFSec/tRNA<sup>sec</sup> complex access to the UGA codon within the ribosomal A-site and subsequent incorporation of Sec instead of translation termination. The addition of Sec also results in an alteration of the SECIS element conformation. Once the tRNA has dissociated from the ribosome, the third protein necessary for the completion of Sec incorporation, rpL30, binds to the now altered SECIS element, releasing SBP2 and allowing its re-association with the ribosome (Kinzy et al., 2005).

### **Quantification of the GPx Enzyme mRNAs**

In addition to tissue Se concentrations and activity of various GPx enzyme tissue activities, comparison of relative GPx mRNA levels is often a desirable method of determining Se status. However, as previously mentioned, not all selenoprotein mRNA levels are affected

similarly by Se depletion (Moriarty et al., 1998) and there appears to be some process of prioritization concerning selenoprotein expression in Se-deficiency. Some selenoprotein mRNAs, such as GPx-4 mRNA, are affected very little by a decrease in Se availability, whereas others, such as GPx-1 mRNA, are affected fairly dramatically. Despite all that is currently known about selenoproteins and their regulation, the specific mechanisms by which this regulatory process occurs are complex and currently poorly understood (Bruzelius et al., 2007).

The utilization of polymerase chain reaction (PCR) in mRNA quantification is considered appropriate due to its high sensitivity, especially in detecting relatively small quantities of product (Moriarty et al., 1998). High detection sensitivity and accuracy are both desirable characteristics when determining relative quantities of mRNA, especially when n-fold differences between samples may be as small as 2; Real-time PCR (Q-PCR) has been shown to consistently provide both of these qualities (Cheng and Maquat, 1993; Moriarty et al., 1998).

Because of its extreme sensitivity to Se status, GPx-1 mRNA is considered an acceptable candidate for comparison of mRNA levels as an indicator of Se status. Research has shown that GPx-1 mRNA levels reliably decrease during Se-deficiency (Christensen and Burgener, 1992; Weiss et al., 1996; Lei et al., 1998; Moriarty, 1998). Glutathione peroxidase-1 mRNA levels have also been used as a parameter contributing to the determination of dietary Se requirement within a specific species and sex during specific developmental stages (Weiss et al., 1996). In fact, comparison of male rat liver GPx-1 mRNA to that of female rats showed that female rats had mRNA levels that were 0.7 fold higher, when both sexes were maintained on a diet containing 0.1 ppm Se as sodium selenite (Weiss et al., 1996). Christensen and Burgener (1992) report a 93-95% reduction in GPx-1 mRNA hybridization in rats fed a Se-deficient diet containing 0.02 ppm Se versus rats fed a diet supplemented with 0.5 ppm Se as sodium selenite.

Weiss et al. (1996) showed that female rats fed a Se-deficient basal diet containing 0.007 ppm Se had liver GPx-1 mRNA levels that were  $11 \pm 1$  % of that observed in female rats fed the basal diet supplemented with 0.1 ppm Se as sodium selenite. Other researchers using rats or mice maintained on a Se-deficient diet for a period of time ranging from 42-135 d have reported decreases in liver GPx-1 mRNA levels in animals of as much as 80-90% (Saedi et al., 1988; Toyoda et al., 1990; Lei et al., 1995). Relative mRNA levels have also been used as an indicator of Se status in some livestock species, but its use is thus far rare. In fact, the only livestock species in which the effect of Se-deficiency on relative mRNA GPx-1 levels has been determined is the pig. Lei et al. (1998) reported a 50% relative decrease in liver GPx-1 mRNA levels for pigs maintained for 5 wks on a Se-deficient diet containing 0.03 ppm Se versus pigs maintained for the same time period on a diet supplemented with 0.2 ppm Se. In contrast, Hostetler et al. (2006) reported finding no significant difference in sow liver GPx-1 mRNA level when sows were fed diets consisting of barley and peas grown in Se-deficient soils and either supplemented or unsupplemented with Se as sodium selenite. Their diets were analyzed as containing 0.05 ppm Se in the Se-deficient diet and 0.39 ppm Se in the Se adequate diet. However, the researchers concluded that, based on the GPx activities and mRNA levels that they observed in the livers of the sows, the sows were actually not Se-deficient, but instead were of marginal Se status. Taken with the work of Lei et al. (1998), this suggests that the margin of Se required to obtain Se-deficient status based on GPx-1 mRNA level may be relatively small in domestic livestock species, requiring extremely low levels of Se to achieve Se-deficiency sufficient to significantly decrease levels of liver GPx-1 mRNA.

## **Selenium and Immunity**

There is ever-increasing evidence suggesting the close relationship of nutritional status to immunity in livestock animals and even humans (Failla, 2003; Chirase, et al.,1991). It has been widely shown that nutrient-deficient hosts often develop more severe pathology than their nutritionally-adequate counterparts when challenged with a viral infection. Antioxidants have long been considered important in immune function and have recently been studied closely to determine their particular role in combating host oxidative stress (Beck, 1999). In particular, the trace mineral Se and the fat-soluble vitamin E have been of considerable interest, as it has been shown that Se deficiencies often result in numerous disease states that are often associated with a concurrent deficit in vitamin E (Turner and Finch, 1991; Wichtel, 1998; Beck and Levander, 2000). Other trace minerals have also been considered to have some antioxidant or immune function, including Fe, Zn, and Cu, and data tend to support the conclusion that inadequate supply of such essential micronutrients is closely related to a decrease in the activities of cells intimately involved in the immune response (Failla, 2003; Hatfield, 2002). A deficiency in Se has been shown to have adverse effects on host immunity and host ability to adequately respond to and clear virus following an infection (Beck, 1999). This decrease in immune effectiveness as a result of selenium deficiency may be due to a decrease in host immune cellular function or even changes in the virus itself brought about by exposure to increased levels of ROS (Beck, 1999).

In addition to direct effects of Se-deficiency on the immune system, anti-oxidation suppression has been shown to influence viral agents. A number of studies have convincingly demonstrated that a non-virulent strain of the *Picornavirus* Coxsackievirus B3 will become a virulent strain of the virus in Se-deficient mice. When infected with a non-virulent strain of

Coxsackievirus B3, Se-deficient mice develop cardiomyopathy. (Beck, 1994a; Beck 1994b; Beck 1994c; Beck 1995; Beck 1998). Furthermore, when virus isolated from the Se-deficient mice was used to infect Se-adequate mice, the Se-adequate mice then too developed severe cardiomyopathy. These results demonstrated that replication of a normally benign virus in a Se-deficient host could result in a virus with an altered genotype, such that increased pathogenicity occurred (Beck, 1999). The same result has been achieved by infecting Se-deficient mice with influenza virus (Beck, 2001). Interestingly, it was shown that the Se-deficiency had no effect on antibody response (Beck, 2001), suggesting that antibody titers alone may not be an adequate means for determining virulence or host ability to fight infection. Thus, Se-deficiency may prove to be a double-edged sword and further research into this area must be conducted to determine the extent to which Se-deficiency contributes to infectious disease.

## **CHAPTER III**

### **THE INFLUENCE OF SE DEFICIENCY IN HOLSTEIN DAIRY CALVES ON TISSUE SE CONCENTRATION, GPX-1 AND 3 ACTIVITY, AND RELATIVE LEVELS OF LIVER GPX-1 MRNA**

#### **Introduction**

There has been considerable research concerning the effect of induced Se-deficiency on tissue Se concentration and GPx-1 and 3 activity in cattle of all sexes and ages. However, to our knowledge there is no data currently available concerning the effect of Se status on relative liver GPx-1 mRNA levels in growing calves. Given the considerable differences in results observed by various laboratories in a wide variety of species, we believe that it is not only necessary but also prudent to both establish ranges of GPx-1 and 3 activities for our laboratory and study the effect of Se-status on liver GPx-1 mRNA in growing calves. We hope to then use this data to further research the effects of Se-deficiency on oxidative stress in growing calves.

#### **Materials and Methods**

##### **Experimental Design**

The LSU Agricultural Center Animal Care and Use Committee approved all animal procedures. Male Holstein calves (n=17), with a mean body weight of 42.96 kg, were purchased within one week after birth from the Southeast Research Station, Franklinton, LA. All calves were born between September, 2006 and December, 2006. Calves were housed individually at the Louisiana State University Agricultural Center Central Station, Baton Rouge, LA, in pens equipped with stainless steel bowl-waterers. Upon arrival, calves were administered 6 cc of Florfenicol subcutaneously and their navels dipped in 7% iodine solution. Each calf was then assigned an individual pen. Calves were fed a commercially available milk replacer (NutraBlend, Neosho, MO) containing 20% protein and 20% fat at 10% of birth weight twice daily until 21 d of age, at which time they were randomly assigned to either a Se-deficient (n=7) or Se-adequate

(n=7) starter diet. In addition, at this time 3 calves were euthanized for collection of baseline liver samples. Diet assignment was determined by pen location; pens were arranged so that Se-adequate and Se-deficient animals were housed adjacent to one another in alternating fashion, both lengthwise down the barn and across the center isle dividing the barn. This arrangement was implemented in order to account for possible confounding variation in barn temperature, humidity, and availability of sunlight. Until 28 d of age, calves received 50% of their diet as commercial milk replacer and the rest as their respective starter diet. Beginning at 28 d of age calves were completely weaned to the starter phase of one of two diets:

- 1) Torula yeast basal diet containing no added Se (n = 7)
- 2) Torula yeast basal diet containing 0.1 ppm Se as sodium selenite (n = 7)

Diets were fed in three phases (Reffett et al., 1988) (Table 3.1) and all calves remained on the initially-assigned Se-deficient or Se-adequate diet until trial completion. Calves were fed and disappearance measured and recorded twice daily.

To ensure that exposure to exogenous sources of Se were minimal, water samples were collected from each of the automatic waterers and analyzed for Se concentration. The analyzed Se content of the water samples was less than 0.005 ppm Se. Analysis of calf diets showed that the Se adequate diet in phase 1 contained  $0.158 \pm 0.034$  ppm Se, while the Se-deficient diet contained  $0.025 \pm 0.008$  ppm Se.

Selenium status of calves was determined by cytosolic GPx-1 and plasma GPx-3 activity. Jugular blood was collected every 28 d beginning at 28 d of age until trial completion. Calves were weighed upon arrival at the Central Research Station barn. Calves were then weighed on 14 d of age and again every 14 d thereafter. Trial termination for each calf occurred upon reaching

180 d of age. In addition, 2 calves in the Se adequate treatment group died of bloat between 140 and 168 d of age.

As previously stated, three calves were euthanized via intravenous Phenobarbital injection at 21 d of age and liver tissue was collected and snap-frozen in liquid nitrogen and stored at -80° C in order to obtain a baseline (BSL) measurement for the group. Calves from each treatment (n=4) were then euthanized at 180 d of age for liver tissue collection and analysis. Liver tissue was wrapped in labeled aluminum foil and snap frozen in liquid nitrogen for storage at -80°C. Messenger RNA was then isolated from each tissue sample for Q-PCR analysis and comparison.

**Table 3.1.** Composition of Torula yeast based diet fed to calves, from Reffett et al., 1988.

Ingredient	%		
	Starter Diet (56d)	Phase 2 (84d)	Phase 3 (12d)
Torula Yeast <sup>a</sup>	30.0	25.0	20.0
Cottonseed Hulls	20.0	30.0	30.0
Dextrose <sup>b</sup>	10.0	4.0	---
Cornstarch <sup>b</sup>	35.0	35.0	44.3
Corn Oil <sup>b</sup>	3.0	3.0	3.0
Mineral/Vitamin Mix <sup>c,d</sup>	2.0	2.0	1.7
Urea	---	1.0	1.0

<sup>a</sup> Provesta Flavor Ingredients, Hutchinson, MN.

<sup>b</sup> International Ingredients Corporation, St. Louis, MO.

<sup>c</sup> Contained the following in mg/kg diet: Ca, 150,000; P, 60,000; Na, 85,000; Mg, 25,000; K, 10,000; Co, 20; Cu, 2500; I, 200; Mn, 4,000; Zn, 6,000.

<sup>d</sup> Contained the following per kg of diet: retinyl acetate, 136,079 IU; cholecalciferol, 13,608 IU; α-tocopherol acetate, 136 IU.

### **Measurement of Diet Se Concentration**

Diets were analyzed for Se concentration by neutron activation at the University of Missouri-Columbia Research Reactor Center. Nine replicates were performed in the analysis of each sample so that the mean obtained from analysis did accurately reflect the Se concentration.

### **Collection and Processing of Blood**

Blood was collected from each calf every 28 d beginning at 28 d of age. Approximately 10 mL of blood were collected in 10 mL heparinized Vacutainer tubes (BD Vacutainer Systems, Franklin Lakes, NJ) via jugular venipuncture. Each blood sample was then centrifuged at 2,000 x g and 4° C for 10 min. Plasma was removed from each sample and placed into a labeled 5 mL Falcon® tube (BD Biosciences, San Jose, CA) and stored at -80° C. The remaining packed erythrocytes were then washed in ice cold phosphate buffered saline and centrifuged 10 min at 2,000 x g at 4° C for a total of three washes. Packed erythrocytes were then pipetted into individually labeled 1.5 mL microcentrifuge tubes and stored at -80° C.

### **Measurement of Plasma GPx-3 Activity**

Because of the relatively quick turnover of plasma proteins and other cells, plasma GPx-3 activity is an indicator of short-term Se status, providing a relatively quick picture of an animal's immediate selenium condition (Gerloff, 1992). Plasma GPx-3 activity was determined using Glutathione Peroxidase Activity Assay Kits (Cayman Chemical Corporation, Ann Arbor, MI) and the ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The Gen 5 data analysis software (BioTek Instruments, Inc., Winooski, VT) was used for data reduction and absorption analyses. Briefly, 100 µL of Assay Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA), 50 µL of Co-Substrate Mixture (a lyophilized powder consisting of NADPH, glutathione, and glutathione reductase), and 20 µL of sample plasma were pipetted, in

triplicate per sample, into a 96 well plate, for a total volume per well of 170  $\mu\text{L}$ . Three controls were also utilized: a blank, consisting of glass-filtered water, a negative enzyme control into which no glutathione peroxidase was added, and a positive control containing 20  $\mu\text{L}$  of supplied purified bovine glutathione peroxidase. Each control was performed in triplicate within each plate that was analyzed in order to account for differences between plates. Each plate was incubated at 25° C and absorbance at 340 nm measured by the plate reader at time zero and then at one min intervals for 6 min, for a total of seven absorbance readings. GPx activity was calculated for each individual well by first determining the change in absorbance at 340 nm ( $\text{Abs}_{340}$ ) per min. Briefly, for each individual well, two points were selected on the linear portion of the resulting curve and the change in absorbance over that time period was calculated using the following equation:

$$\Delta A_{340}/\text{min} = (\Delta A_{340} @ \text{Time 2} - \Delta A_{340} @ \text{Time 1}) / \text{Time 2 (min)} - \text{Time 1 (min)},$$

where  $\Delta A_{340}$  was the absorbance at each respective time and Time 1 was the first chronological time in min and Time 2 was the second chronological time in min. The  $\Delta A_{340}/\text{min}$  was then also calculated for the non-enzymatic control wells and this rate subtracted from that of the sample wells. Activity was then determined using the NADPH extinction coefficient of  $0.00373 \mu\text{M}^{-1}$ , which is the value adjusted for the 0.6 cm path length of the solution in the well from the actual value of  $0.00622 \mu\text{M}^{-1}$ . Activity was calculated using the following formula:

$$\text{GPx activity} = (|\Delta A_{340}/\text{min}| / 0.00373 \mu\text{M}^{-1}) \times (0.19 \text{ ml} / 0.02 \text{ ml}) = \text{nmol}/\text{min}/\text{mL},$$

where  $|\Delta A_{340}/\text{min}|$  was the absolute value of the calculated absorbance at 340 nm per min and one enzyme unit was defined as the amount of enzyme that will cause the oxidation of 1 nmol NADPH per min.

Glutathione Peroxidase-3 activity was expressed as EU per gram protein, where 1 EU was equivalent to the amount of GPx activity required to oxidize 1  $\mu\text{mol}$  of NADPH per 1 min. Plasma protein concentrations were determined using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad Laboratories, Inc., Hercules, CA), an assay first described by Bradford (1976) in which the dye agent, Coomassie Brilliant Blue G-250 is used to bind solubilized protein. Plasma samples were diluted 1:50 in water before assaying. Briefly, a standard curve was constructed using 2 mg/mL bovine serum albumin (BSA) in which seven concentrations ranging from 0  $\mu\text{g/mL}$  BSA to 2000  $\mu\text{g/mL}$  BSA were used. Standards were analyzed in triplicate. Samples were then diluted 1:50 using glass-filtered  $\text{H}_2\text{O}$  and 20  $\mu\text{L}$  were pipetted into each of two disposable cuvettes (Bio-Rad Laboratories, Inc., Hercules, CA). To initiate the reaction, 1 mL of Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA) was added to each cuvette and allowed to incubate for 5 min after gentle inversion. Absorbance was measured at 595 nm using the Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) and concentrations were calculated using the previously constructed standard curve. Thus, using protein concentrations, all GPx activities were expressed per g protein.

#### **Measurement of Cytosolic GPx-1 Activity in Erythrocytes**

Due to the slow turnover of erythrocytes, with which 98% of GPx activity is associated (Scholtz and Hutchinson, 1979), and the fact that GPx-1 is incorporated into erythrocytes during erythropoiesis, GPx-1 activity in erythrocytes is considered an indicator of long-term Se status (Gerloff, 1992). Cytosolic GPx-1 activity was determined using Glutathione Peroxidase Activity Assay Kits (Cayman Chemical Corporation, Ann Arbor, MI) and the ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT), as previously described. Thawed erythrocytes were then lysed in ice-cold glass-filtered water to a 1:4 dilution, followed by 1:100

dilution of the lysate in sample buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/mL BSA). The assay procedure and activity calculations were the same as previously described for determination of Plasma GPx-3 activity.

Erythrocyte GPx-1 activities were expressed as enzyme units per gram of hemoglobin, where 1 EU was equivalent to the amount of GPx activity required to oxidize 1  $\mu$ mol of NADPH per 1 min. Hemoglobin was measured using Quantichrom™ Hemoglobin Assay Kits (BioAssay Systems, Hayward, CA). Briefly, blank and standard wells were made in triplicate for each plate performed by pipetting 50  $\mu$ L of water for the standards and 50  $\mu$ L of provided standard solution containing 100 mg/dL hemoglobin in 0.02% tartrazine, followed by the addition of 200  $\mu$ L water into each of three wells. Blood samples were first diluted 100 fold in ice-cold distilled water. Samples were analyzed in duplicate by pipetting 50  $\mu$ L of sample into each of two wells, followed by 200  $\mu$ L of Reagent (0.40% sodium hydroxide and 2.60% Triton X-100), provided in the kit. Plates were then incubated for 5 min at approximately 27° C. Absorbance at 400 nm was then read using a Power Wave Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Absorbance data were collected by the Gen5 Software System (BioTek Instruments, Inc., Winooski, VT). Hemoglobin concentration (mg/dL) was calculated with the following equation:

$$(\text{Abs}_{400} \text{ sample} - \text{Abs}_{400} \text{ blank} / \text{Abs}_{400} \text{ standard} - \text{Abs}_{400} \text{ blank}) * 100 * n,$$

where “n” was the dilution factor.

### **Measurement of Cytosolic GPx-1 Activity in Liver Tissue**

Cytosolic GPx-1 in liver tissue was again determined using Glutathione Peroxidase Activity Assay Kits (Cayman Chemical Corporation, Ann Arbor, MI) and the ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Liver tissue collected

from three BSL calves, four Se adequate calves, and four Se-deficient calves and snap-frozen in liquid nitrogen was used to determine GPx-1 activity. Briefly, approximately 1g of each liver sample was individually ground under liquid nitrogen with a frozen mortar and pestle without allowing the samples to thaw. The finely ground sample was then placed into a 14 mL Falcon<sup>®</sup> tube (BD Biosciences, San Jose, CA) and 10 mL of cold buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM DTT) were added to the sample and mixed thoroughly. The samples were then each centrifuged at 10,000 x g for 15 min at 4° C and the supernatant removed and stored at -80° C for no longer than one month. Hemolysate dilutions of 1:400 were prepared using sample buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/mL BSA). The liver hemolysate was then used to determine liver GPx-1 activity using the Cayman Chemical Glutathione Peroxidase Activity kit (Cayman Chemical Corporation, Ann Arbor, MI), but with some minor modifications. Briefly, 70 µL of Assay Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA), 50 µL of Co-Substrate Mixture (a lyophilized powder consisting of NADPH, glutathione, and glutathione reductase), 20 µL Double Strength Drabkin's Reagent (Potassium cyanide/Potassium ferricyanide), 10 µL 0.01 M NaN<sub>3</sub>, and 20 µL of sample hemolysate were pipetted, in triplicate per sample, into a 96 well plate placed on ice, for a total per well volume of 170 µL. Four controls were also utilized: a blank, consisting of glass-filtered water, a blank consisting only of reagent and to which no hydrogen peroxide was added, a negative enzyme control into which no glutathione peroxidase was added, and a positive control containing 20 µL of supplied purified bovine glutathione peroxidase. Each control was performed in triplicate within each plate that was analyzed in order to account for differences between plates. To initiate the reaction, 20 µL of 12 mM H<sub>2</sub>O<sub>2</sub> was pipetted into each well used and then the plate was incubated at 25° C and absorbance at 340 nm measured by the plate reader at time zero and then

at one min intervals for 10 min, for a total of eleven absorbance readings. Liver GPx-1 activities were then calculated as outlined for GPx-3. GPx-1 activity in liver tissue was expressed as enzyme units per gram protein, and tissue protein concentrations were determined on 1:10 dilutions of liver tissue hemolysate in water using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad Laboratories, Inc., Hercules, CA) as previously described.

### **Measurement of Liver Tissue Se Concentration**

Liver tissue Se concentration was determined via the method of Martin and Williams (1989), with some changes. Briefly, liver samples were dried at 60° C in a drying oven and weighed every 12 h for 36 h until completely dry. Approximately 0.5 g of tissue from each sample was weighed in duplicate into Teflon<sup>®</sup> microwave tubes and 4 mL of 70% HNO<sub>3</sub> were added to each tube. Samples were allowed to pre-digest for 12 h before digestion. Liver samples were then digested in a MARS5 microwave digester (CEM Corporation, Matthews, NC) at 1,200 watts on maximum power with a 15 min ramp time followed by 15 min of digestion at 200° C.

After cooling, digested samples were brought to 10 mL total volume with deionized, distilled water and stored in 14 mL polystyrene Falcon<sup>®</sup> tubes (BD Biosciences, San Jose, CA) until processing. Selenium concentration was determined by the method of GFAAS using a Perkin Elmer Zeeman 5100 Graphite Furnace Spectrophotometer (Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA) at 196 nm.

### **Isolation of mRNA from Liver Tissue**

Total mRNA was isolated from frozen liver tissue using the Dynabeads<sup>®</sup> mRNA DIRECT Isolation Kit (DynaL Biotech, Inc., Lake Success, NY, USA). Approximately 30 to 50 mg of frozen liver tissue stored at -80° C was ground in liquid nitrogen using an autoclaved mortar and pestle frozen in liquid nitrogen. The tissue was not allowed to thaw at any time before

the addition of 1,250  $\mu$ L of lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, and 5 mM dithiothreitol) to the sample. The sample was then homogenized for 3 to 5 min in the lysis/binding buffer using a pair of autoclaved hemostatic forceps previously wiped with RNase Away (Molecular Bio-Products, Inc., San Diego, CA). The lysed sample was then forcibly passed 3 to 5 times through a 21 gauge needle using a 3 cc sterile syringe in order to shear the DNA. After centrifuging the sample for 60 s, the supernatant was removed and added to 250  $\mu$ L of pre-washed Dynabeads Oligo (dT)<sub>25</sub>. The sample and beads were allowed to hybridize for 5 min with continuous mixing in a roller mixer in order to give the mRNA poly-A tail time to anneal to the Dynabead (dT)<sub>25</sub>. The microcentrifuge tube containing the mixture was then placed onto the Dynal MPC-E-1 magnetic separator for 2 min and the resulting supernatant discarded. The remaining beads-mRNA complex was then washed twice in 1 mL of washing buffer A (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1 mM EDTA, and 0.1% lithium dodecylsulfate) followed by a single wash in 1 mL of washing buffer B (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, and 1 mM EDTA). The beads-mRNA complex was then washed in an additional 40  $\mu$ L of washing buffer B. After discarding washing buffer B, the mRNA was eluted by adding 25  $\mu$ L of 10 mM Tris-HCl to the beads-mRNA complex and incubation for 2 min in a 75° C water bath. The microcentrifuge tube containing the eluted RNA and beads was then placed immediately onto the magnet and the free mRNA was removed and added to a labeled nuclease-free 0.5 mL centrifuge tube. The mRNA was then kept on ice for immediate cDNA synthesis.

### **Reverse Transcriptase cDNA Synthesis**

Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Briefly, 12.5  $\mu$ L of isolated template mRNA was

added to 4  $\mu$ L of 5x iScript reaction mix, 2.5  $\mu$ L nuclease-free water, and 1  $\mu$ L reverse transcriptase in a PCR tube for a total volume of 20  $\mu$ L. A no reverse transcriptase control reaction containing 12.5  $\mu$ L isolated template mRNA, 4  $\mu$ L 5x iScript reaction mix, 2.4  $\mu$ L nuclease-free water and 1  $\mu$ L autoclaved water was also performed. Reverse transcriptase PCR (RT-PCR) was performed using an iCycler ThermalCycler (Bio-Rad Laboratories, Inc., Hercules, CA) at 25° C for 5 min, 42° C for 30 min, and a 5 min denaturing step at 85° C with a final hold temperature of 4° C.

### **Primer Design for PCR Amplification**

Primers for PCR amplification of GPx-1 cDNA were designed based on a published bovine nucleotide sequence (Mullenbach et al., 1988; GenBank Accession No. X13684, 828 nucleotides) using the Beacon Designer 4.0 software program (PREMIER Biosoft International). Primers for PCR amplification of Poly-A polymerase cDNA were based on a published bovine nucleotide sequence (Raabe et al., 1991; GenBank Accession No.X63436, 3431 nucleotides). Primers were synthesized by Life Technologies (Invitrogen, Carlsbad, CA) (Table 3.2).

**Table 3.2.** Primers for amplification of GPx-1 and Poly-A cDNA.

<b>Gene</b>	<b>Accession Number</b>	<b>Primer Sequence</b>	<b>Amplicon Length (bp)</b>
Poly-A	X63436	Sense AAGCAACTCCATCAACTACTG	169
		Antisense ACGGACTGGTCTTCATAGC	
GPx-1	X13684	Sense AACGCCAAGAACGAGGAG	204
		Antisense GGACCAGGTGATGAACTTAGG	

### **Validation of Primers and RNA Isolation**

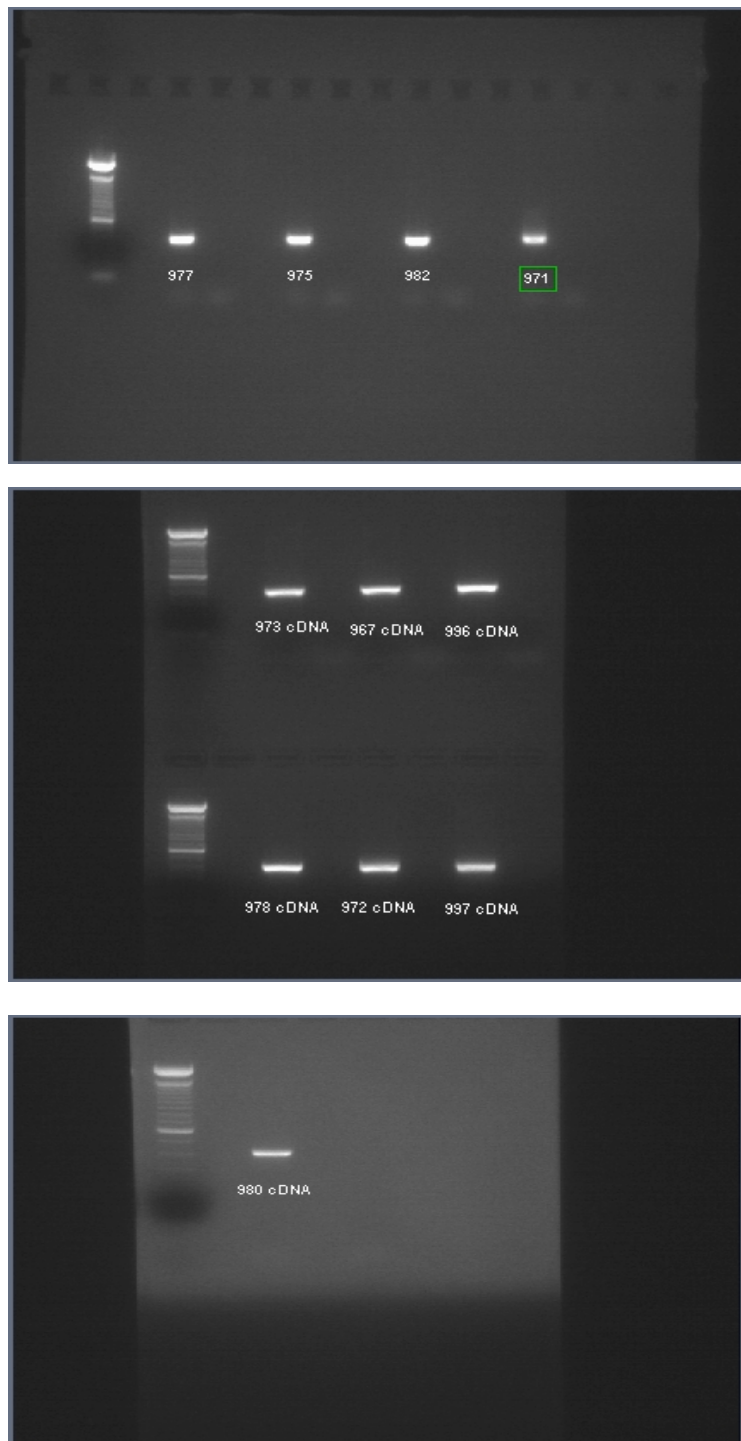
Based on previous work (Wrenzycki, 1998), Poly-A polymerase (Poly-A) was chosen as an internal control gene for this experiment, as its expression and abundance are unaffected by Se status (Lei et al., 1995). The primers for this gene and GPx were validated by performing a

PCR amplification using cDNA from the calibrator, which was cDNA combined from the liver tissue samples taken from the three BSL calves slaughtered at 21 d of age. Each reaction contained 5  $\mu$ L of 10x PCR buffer, 1  $\mu$ L dNTP (10  $\mu$ L dGTP, 10  $\mu$ L dCTP, 10  $\mu$ L dATP, 10  $\mu$ L dTTP, and 60  $\mu$ L autoclaved water), 1  $\mu$ L forward primer (either GPx or Poly-A), 1  $\mu$ L reverse primer (either GPx or Poly-A), 39  $\mu$ L autoclaved water, 1  $\mu$ L template cDNA, and 2  $\mu$ L Jumpstart™ REDTaq™ DNA Polymerase (Sigma, Saint Louis, MO) for a total reaction volume of 50  $\mu$ L. A working master mix for each primer set was first made, consisting of all reaction components with the exception of the template cDNA. This mix was then pipetted into individual PCR tubes and 1  $\mu$ L cDNA added to each PCR tube for a total reaction volume of 50  $\mu$ L. Amplification was performed in the iCycler Thermalcycler (Bio-Rad Laboratories, Inc., Hercules, CA) with a 95° C initial denaturing step for 1 min, 35 cycles of 30 s denaturation at 95° C, 30 s annealing at 56° C, and 30 s extension at 72° C, followed by a 4 m final extension at 72° C and a final hold temperature of 4° C. Gel electrophoresis was then performed by loading 25  $\mu$ L of each sample into individual wells of a 2% agarose gel. A 100 bp ladder added to the same gel was used to confirm the size of the resulting amplicon for each gene of interest. The presence of GPx mRNA in all isolated samples was then confirmed using the same protocol as discussed previously (Figure 3.1).

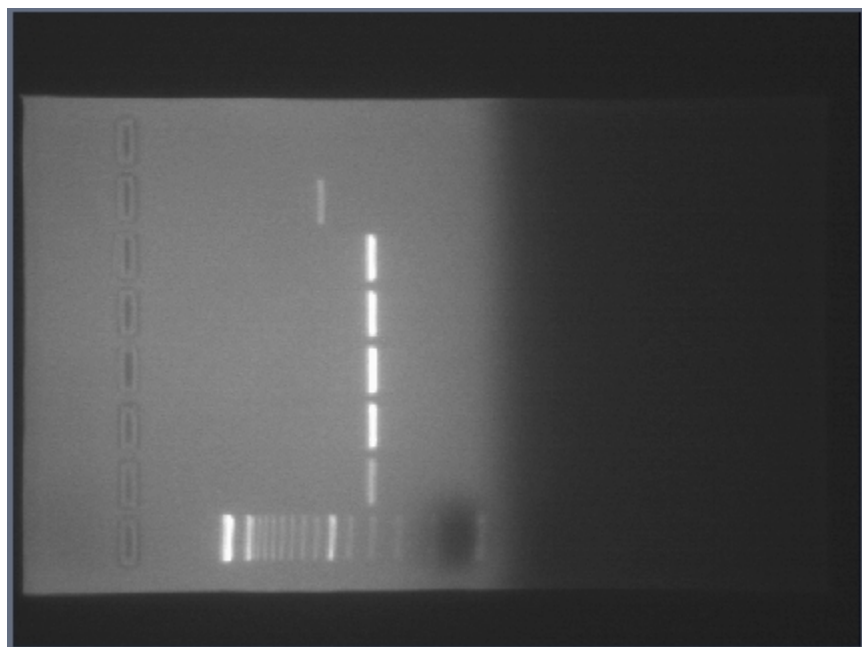
### **Optimization and Validation of Quantitative Real Time PCR (Q-PCR)**

Optimization of primer conditions is a necessary part of ensuring that primers amplify only target cDNA during PCR. Primer annealing temperature was optimized by utilizing an annealing temperature gradient consisting of five temperatures from 50° C to 65° C. Gel electrophoresis in a 2% agarose gel was then used to determine the best annealing temperature

based on amplicon signal intensity. An annealing temperature of 56° C was chosen based on the  
apparent quantity



**Figure 3.1.** Gel of bovine GPx cDNA in samples and individual baseline calves. The no reverse transcriptase controls are located in the lane to the right of each sample. The 100 bp ladder is in the first well, to the left of samples in all three gels.



**Figure 3.2.** Gel of annealing temperature gradient. The amplicon obtained by annealing at 56° C is the fourth well from the bottom of the gel. The first well is a 100 bp ladder.

of amplicon as determined by relative band brightness when electrophoresed and placed into a Gel Dock Eq (Bio-Rad Laboratories, Inc., Hercules, CA) for viewing under ultraviolet light (Figure 3.2).

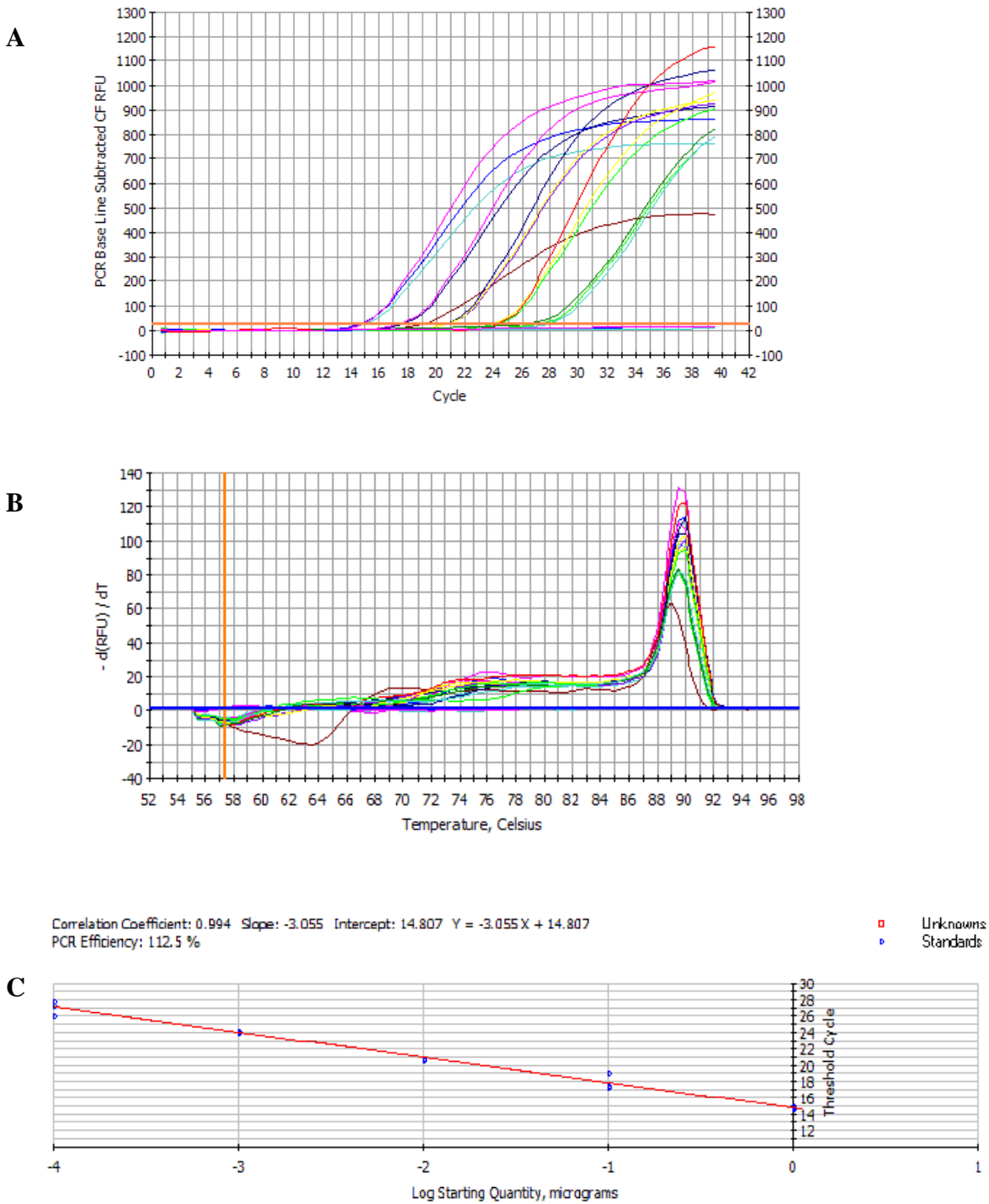
In order to ensure that the chosen primers were indeed quantitative in amplification, ten-fold dilution series were performed with the calibrator, an equal mix of cDNA from the three BSL calves. Both Poly-A and GPx-1 primers were validated in this manner in separate reactions. Ten-fold dilutions were prepared by first adding 2  $\mu$ L of undiluted calibrator cDNA to 18  $\mu$ L of autoclaved water, making a 1:10 dilution. From this dilution, 1:100, 1:1000, and 1:10,000 dilutions were made. In addition, a “no template control” consisting of only autoclaved water and lacking any template cDNA was also made and utilized to ensure that there was no DNAcontamination of the reaction. Each reaction consisted of 12.5  $\mu$ L of SYBR Green (Bio-Rad

Laboratories, Inc., Hercules, CA), 1  $\mu$ L of sense primer for the gene to be amplified, 1  $\mu$ L of anti-sense primer for the gene to be amplified, 9.5  $\mu$ L of autoclaved water, and 1  $\mu$ L of cDNA from one of the dilutions for a total reaction volume of 25  $\mu$ L. A master mix containing all reactants with exception of template cDNA and dilutions was first made and 96  $\mu$ L of said master mix was pipetted into six 0.5  $\mu$ L microcentrifuge tubes. Into each tube was then pipetted 4  $\mu$ L of one of the dilutions. The no template control reaction tube received 4  $\mu$ L of autoclaved water. The contents of each tube were then pipetted in triplicate into half of a 96-well plate, for a total volume of 25  $\mu$ L per well. The plate was then placed into an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) and a two-step amplification was performed with an initial denaturation step for 3 min at 95° C for 1 cycle, followed by 40 cycles of a two step process consisting of denaturation for 10 s at 95° C and annealing for 45 s at 56° C. Florescence was measured during this time and real-time analysis performed using the My iQ software system (Bio-Rad Laboratories, Inc., Hercules, CA). This was followed by 1 cycle of denaturation at 95° C for 1 min, one cycle of annealing at 56° C for 1 min, and 80 cycles at 55° C. After cycle 2 at 55° C, the temperature was increased by 0.5° C intervals and melt curve data collected for the duration of the cycles. A final hold temperature of 10° C was then maintained. From each analysis a standard curve, melt curve, and dilution Ct values of the ten-fold dilutions were created and the efficiency and correlation coefficient provided by the My iQ Reverse Transcription PCR Detection software system (Bio-Rad Laboratories, Inc., Hercules, CA) (Figure 3.3). All of the target genes in both the samples and calibrator had efficiencies between 80 and 120%, which is the range considered acceptable for quantitative PCR. Correlation coefficients for all target genes were also acceptable, being as close to 1.0 as possible.

### **Real Time Q-PCR of GPx-1 and Poly-A Polymerase**

Real time PCR was performed to measure the relative amount of GPx, the gene of interest, and Poly-A polymerase, the internal control, mRNA in each of the eight liver samples and the calibrator. Each reaction consisted of 12.5  $\mu\text{L}$  SYBR Green (Bio-Rad Laboratories, Inc., Hercules, CA), 1  $\mu\text{L}$  of sense primer for the gene to be amplified, 1  $\mu\text{L}$  antisense primer for the gene to be amplified, 9.5  $\mu\text{L}$  autoclaved  $\text{H}_2\text{O}$ , and 1  $\mu\text{L}$  of the cDNA to be amplified for a final reaction volume of 25  $\mu\text{L}$  per well. Two master mixes were made containing all reactants with exception of template cDNA, one for each gene of interest, and Q-PCR was performed for both genes in all samples, including the calibrator. Again, a “no template control” consisting of only autoclaved water and lacking any template cDNA was also made for both GPx and Poly-A, which was utilized to ensure that there was no DNA contamination of the reaction. Into each of ten 0.5  $\mu\text{L}$  microcentrifuge tubes per gene of interest was pipetted 96  $\mu\text{L}$  of master mix, followed by 4  $\mu\text{L}$  of sample cDNA, one sample per microcentrifuge tube per gene. The no template controls each received 4  $\mu\text{L}$  of autoclaved water. The contents of each tube were then pipetted in triplicate into a 96-well plate, for a total volume of 25  $\mu\text{L}$  per well. The plate was placed into the MyIQ ThermalCycler (Bio-Rad Laboratories, Inc., Hercules, CA) and a two-step amplification was performed, as previously discussed in the validation of the Q-PCR reaction.

SYBR Green fluorescence was detected by the MyIQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Data were used to calculate the quantity of mRNA based on the method for relative quantification in Q-PCR based on previous work by Pfaffl (2001), in which quantities of mRNA are expressed as n-fold differences relative to a calibrator. The calibrator used for this experiment consisted of cDNA from the combined liver



**Figure 3.3.** Dilution Ct values, melting curves, and standard curve obtained with primers for GPx-1 amplification in calibrator sample. Five 10-fold dilutions (undiluted, 1:10, 1:100, 1:1000, and 1:10,000) of calibrator cDNA were used for the generation of **(A)** dilution Ct values, **(B)** melting curves, and **(C)** a standard curve.

samples collected from three 21 d old calves considered as baselines, euthanized prior to starting on a Se-adequate or Se-deficient diet.

Poly-A polymerase was used as the reference gene for all samples, including the calibrator.

Relative transcription values were then calculated by first using the threshold cycle ( $C_T$ ) values of the reference gene to normalize the target gene signals for each sample, resulting in the  $\Delta C_{T,T}$  for the target gene and the  $\Delta C_{T,R}$  for the reference gene. The  $\Delta C_{T,T}$  was calculated by subtracting the  $C_T$  value for the target gene in the calibrator from the  $C_T$  value for the target gene in each sample. Likewise, the  $\Delta C_{T,R}$  was calculated by subtracting the  $C_T$  value for the reference gene in the calibrator from that of the reference gene in each sample. The amount of target gene amplicon relative to the calibrator was then calculated using the following formula:

$$\text{n-fold difference} = \text{Efficiency Target Gene}^{\Delta C_{T,T}} / \text{Efficiency Reference Gene}^{\Delta C_{T,R}} .$$

As a result, all mRNA quantities were expressed as the n-fold difference related to the calibrator, normalized to the reference gene.

### **Statistical Analysis**

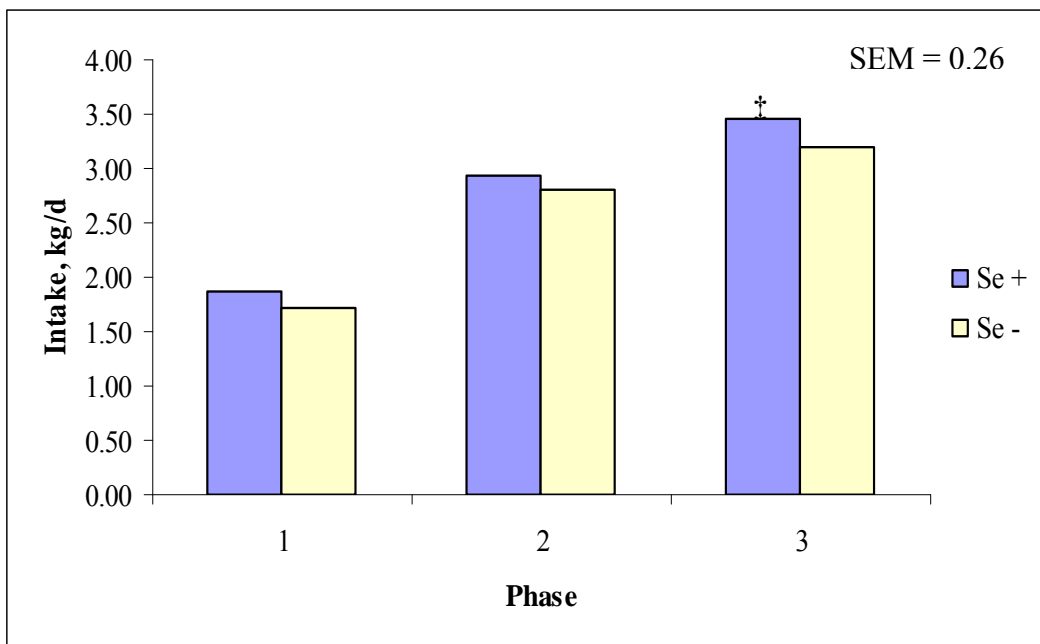
Statistical analyses were performed on all data to determine statistical significance. Response variables of interest were average daily gain (ADG), average daily intake (ADI), liver tissue Se concentration, RBC-GPx activity, GPx-3 activity, liver GPx-1 mRNA, and relative liver GPx-1 mRNA n-fold difference. Calf was considered the experimental unit for all analyses and significance was determined at the 0.05 level. Calf ADG, ADI, RBC-GPx activity, and GPx-3 activity were considered to be repeated measures and were analyzed using the Proc Mixed procedure in SAS (SAS Institute, Cary, SC). Liver tissue Se concentrations, liver GPx-1 activity, and relative liver mRNA data were treated as a completely randomized ANOVA and were analyzed using the Proc Mixed procedure of SAS (SAS Institute, Cary, SC). Regression and correlation analyses were performed on relative mRNA levels, RBC-GPx activity, and GPx-3

activity using the Proc Reg and Proc Corr procedures of SAS (SAS Institute, Cary, SC), respectively.

## Results and Discussion

### Growth and Intake

Supplemental Se inclusion had no effect on ADI at any time during the experiment. The mean ADI for diet phase 1, which occurred from 28 d of age until 83 d of age, was  $1.86 \pm 0.07$  kg/d. During phase 2, which lasted from 84 d of age until 167 d of age, the mean ADI was  $2.88 \pm 0.07$  kg/d. Average daily intake during phase 3, which lasted from 168 d until trial completion at 180 d of age, was  $3.30 \pm 0.08$  kg/d (Figure 3.4).



‡The mean of 5 calves.

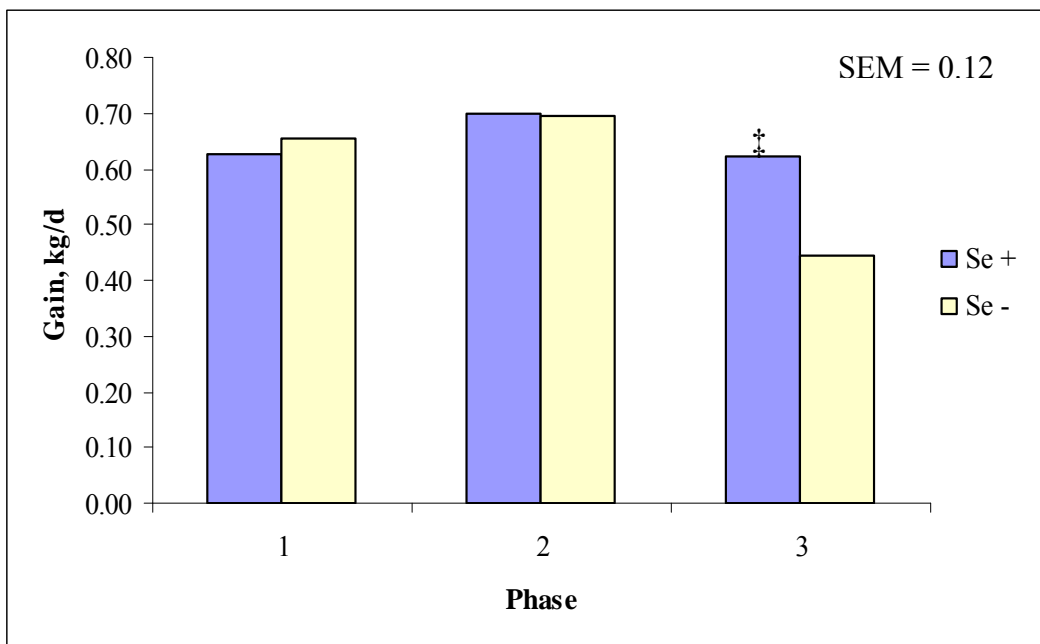
**Figure 3.4.** Average daily intake (kg/d) by phase for calves fed diets supplemented or unsupplemented with Se.

Likewise, growth was unaffected by dietary treatment (Figure 3.5), which was in agreement with findings in previous research in swine, cattle, and poultry (Mahan and Parrett, 1996; Lawler et

al., 2004; Payne and Southern, 2005). The ADG during phase 1 was  $0.65 \pm 0.097$  kg/d. Average daily gain during phase 2 was  $0.69 \pm 0.097$  kg/d, while phase 3 ADG was  $0.53 \pm 0.110$  kg/d.

### Liver Se Concentration

Liver Se concentration at d 180 of age for baseline calves and calves maintained on the Se adequate diet were significantly higher than that of calves maintained on the Se-deficient diet, but liver Se concentration was not different between baseline calves and calves maintained on the Se-adequate diet. Mean Se concentration in the livers of baseline calves and those maintained on the Se-adequate diet were  $0.800 \pm 0.105$  ppm and  $0.742 \pm 0.091$  ppm, respectively (dry weight). Mean Se concentration in the livers of calves maintained on the Se-deficient diet was  $0.412 \pm 0.091$  ppm (dry weight) (Figure 3.6).



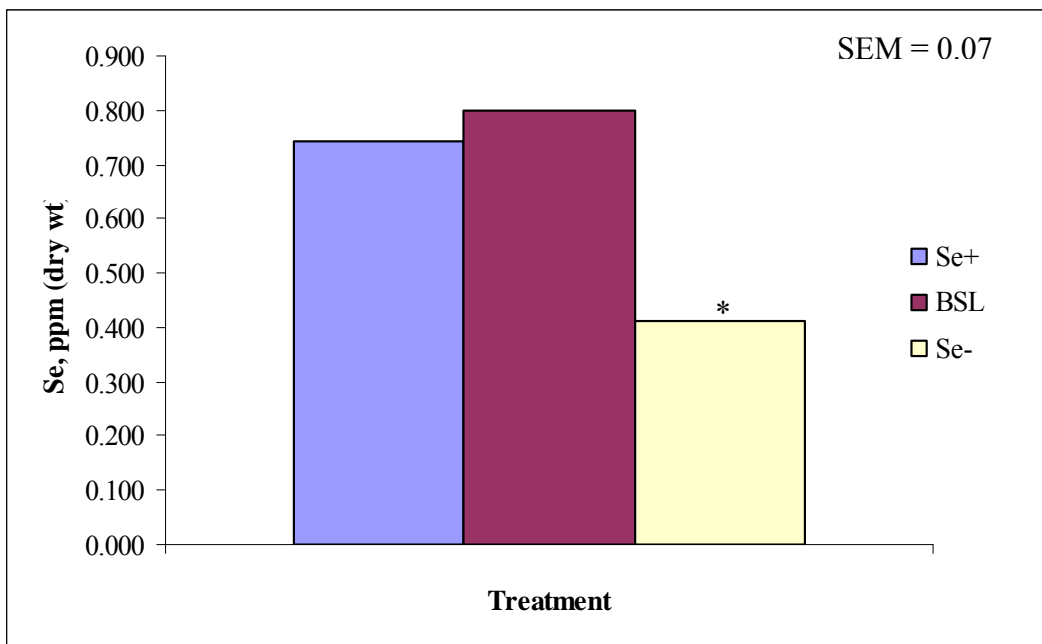
‡ The mean of 5 calves.

**Figure 3.5.** Average daily gain (kg/d) by phase for calves fed diets supplemented or unsupplemented with Se.

The mean liver Se concentration of calves fed the Se-deficient diet was higher than that reported in 8 wk old lambs by Oh et al. (1976), in which lambs fed a torula yeast-based milk diet

containing 0.01 ppm Se had an average liver Se concentration of 0.049 ppm Se on a wet basis, but the mean liver Se concentrations of calves fed the Se adequate diet were similar to lambs fed the torula-based milk diet supplemented with 0.10 ppm Se. Lambs supplemented with 0.10 ppm Se were reported as having an average liver Se concentration of 0.183 ppm Se on a wet basis.

Although Se concentration is generally considered to be higher in muscle tissue (Behne and Wolters, 1983), the concentration of Se in the liver is of considerable importance, as it is reflective of Se status (Abdelrahman and Kincaid, 1995). This is demonstrated by the 1 comparison of the distribution of Se concentration in various tissues and the distribution of GPx-1 activity in those same tissues. While muscle and liver tissue have been reported to contain approximately 39.8 and 31.7% of the Se found in the body respectively, only 6.1 % of GPx-1



\* $P < 0.05$ .

**Figure 3.6.** Mean liver Se concentration by treatment at d 180 of age.

activity is located within muscle tissue while 65.6 % of GPx-1 activity is located in liver tissue (Behne and Wolters, 1983). The liver Se concentration of the sampled calves at d 180 of age

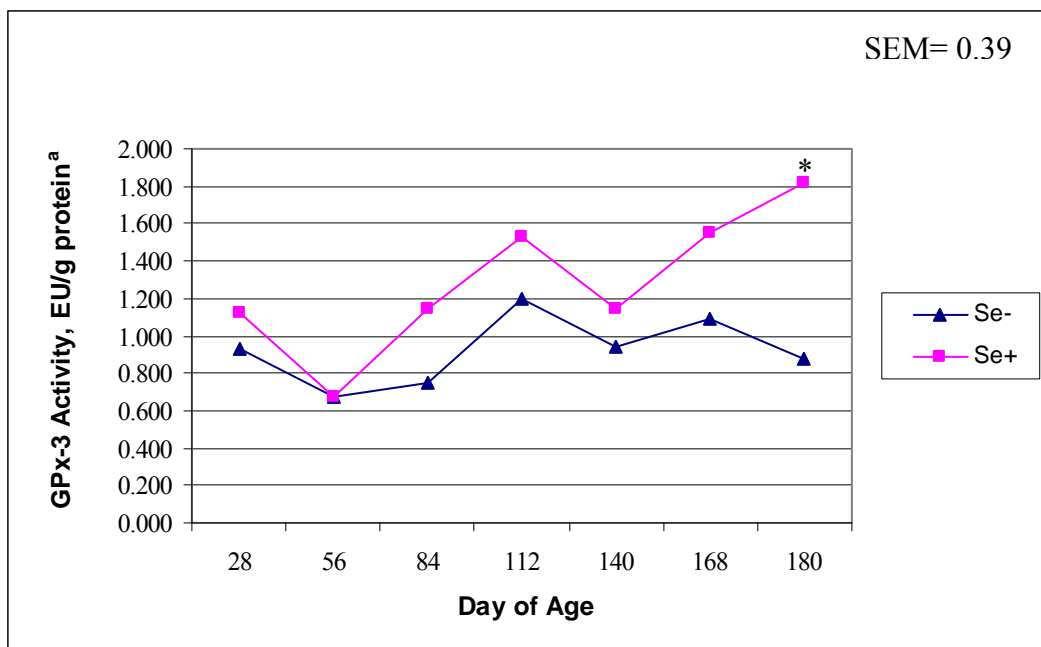
followed the expected trend: calves maintained on the Se adequate diet had, on average, almost 50% higher liver Se concentrations than that of calves maintained on the Se-deficient diet. This was in agreement with the findings of previous research in which mammals of various species maintained on Se-deficient diets were shown to have significantly decreased concentrations of liver Se when compared to that of animals fed a diet adequate in Se (Mahan et al., 1999; Payne and Southern, 2005; Hostetler et al., 2006). However, the Se concentration of Se adequate and deficient treatment groups were lower than the expected range noted by Stowe and Herdt (1992), in which they report the normal liver Se concentration of all species, regardless of age, should be in the range of 1.2 to 2.0 ppm Se on a dry weight basis. The liver Se concentrations in both Se adequate and Se-deficient calves were also lower than values reported by Abdelrahman and Kincaid (1995) in which 42 d old calves born to dams either supplemented with Se via a Se bolus or unsupplemented with Se had a mean liver Se concentration of 2.62 and 1.20 ppm Se on a dry weight basis, respectively.

### **Glutathione Peroxidase-3 and Glutathione Peroxidase-1 Activities**

Plasma GPx-3 activity varied considerably between sampling times throughout the duration of the trial for both treatment groups, but a significant difference ( $P < 0.05$ ) between treatments was not detected until d 180 of age. At this time a decrease of approximately 52% in GPx-3 activity in the Se-deficient group was observed ( $1.79 \pm 0.20$  versus  $0.86 \pm 0.16$  EU/g protein) (Figure 3.7).

The RBC-GPx activity was more reflective of the Se content when compared to GPx-3 activity. While treatment differences in GPx-3 activity were observed on d 180 of age, calves supplemented with Se had higher RBC-GPx activity at d 84 of age and through trial termination ( $P < 0.01$ ). On d 84 of age the mean RBC-GPx activity of calves maintained on the Se adequate diet was  $52.09 \pm 4.61$  EU/g Hb, while the mean RBC-GPx activity for calves fed the Se-deficient

diet was  $24.47 \pm 4.61$  EU/g Hb. This represented approximately a 61% decrease in RBC-GPx activity in those calves maintained on the Se-deficient diet. By d 180 of age the mean RBC-GPx activity of Se-adequate and Se-deficient calves was  $54.98 \pm 6.20$  and  $11.56 \pm 4.95$  EU/g Hb, respectively, representing a 79% decrease in GPx-1 activity in calves maintained on the Se-deficient diet (Figure 3.8).



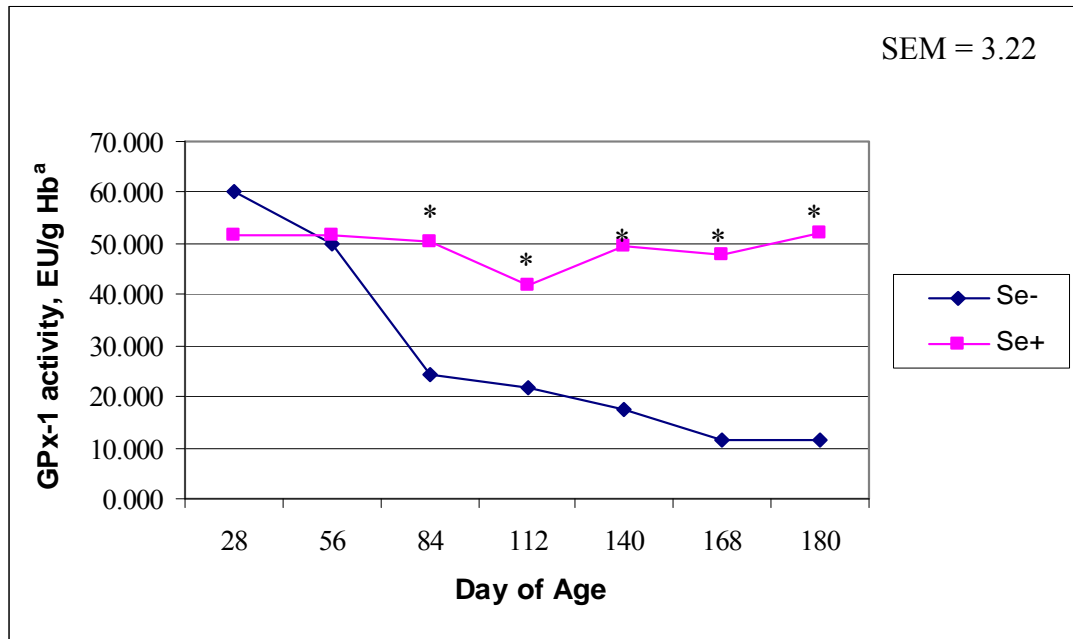
<sup>a</sup> EU= enzyme unit; 1 EU is the amount of GPx activity required to oxidize 1  $\mu$ mol NADPH per min.

\*  $P < 0.05$ .

**Figure 3.7.** Mean GPx-3 activity of Se supplemented and unsupplemented calves by day.

As previously discussed, GPx-3 activity is generally considered to be an indicator of short term Se status. Therefore, the considerable variation observed with respect to the trend in GPx-3 activity for both groups was expected, as day to day feed intake and hydration status may have played a role in affecting GPx-3 activity measurements. In addition, Oh et al. (1976) reported that unlike circulating plasma Se, erythrocytes contain a relatively constant proportion of total blood Se. However, it was surprising that no detectable decrease in GPx-3 activity

occurred before d 180 of age, when the first significant decrease in RBC-GPx activity for calves maintained on the Se-deficient diet was observed by d 84 of age. Erythrocyte GPx-1 activity was not expected to differ significantly until calves were older, based on the 90 to 120 d whole body turnover of erythrocytes (Gerloff, 1992); likewise, GPx-3 activity was expected to decrease in Se deficient calves at an earlier time point than observed. However, the seemingly early decrease in



<sup>a</sup>EU= enzyme unit; 1 EU is the amount of GPx activity required to oxidize 1  $\mu\text{mol}$  NADPH per min.

\*  $P < 0.0001$ .

**Figure 3.8.** Mean erythrocyte GPx-1 activity of Se supplemented and unsupplemented calves by day.

RBC-GPx activity may be explained by considering that RBC-GPx is incorporated into erythrocytes during erythropoiesis (Gerloff, 1992). The time at which the first difference in RBC-GPx activity was observed between treatments actually approximates the half-life of bovine erythrocytes (Oh et al., 1976; Gerloff, 1992) and RBC-GPx activities were analyzed as a mean of all calves within a treatment group. In addition, the considerable variation observed with respect to GPx-3 activity may also be explained as a function of the fact that GPx-3 is

considered a short-term indicator of Se status (Gerloff, 1992). Although calves were limit-fed with regard to the amount of ration they received at each feeding, they were not limited in the amount of time allowed to consume the ration. Not all calves were observed to eat at the same rate; it is possible that calves consuming the ration slowly throughout the day might have maintained higher, more consistent plasma Se levels and therefore higher GPx-3 activity levels if sampled shortly after consuming feed, affecting the overall trend in GPx-3 activity.

Plasma GPx activity for both treatment groups was in agreement with GPx-3 activities previously reported by Rowntree et al. (2004) in beef cows, in which cows either receiving no supplemental Se or a 20 mg Se drench per day had an average GPx-3 activity of 0.92 and 1.34 EU/g protein, respectively. The decrease in GPx-3 activity observed in calves maintained on the Se-deficient diet were in agreement with previous reports in various species in which animals were fed either a diet adequate or deficient in Se, although the actual amounts of GPx-3 activity varied considerably from species to species (Weiss et al., 1996; Lei et al., 1998; Enjalbert, 1999; Mahan et al., 1999).

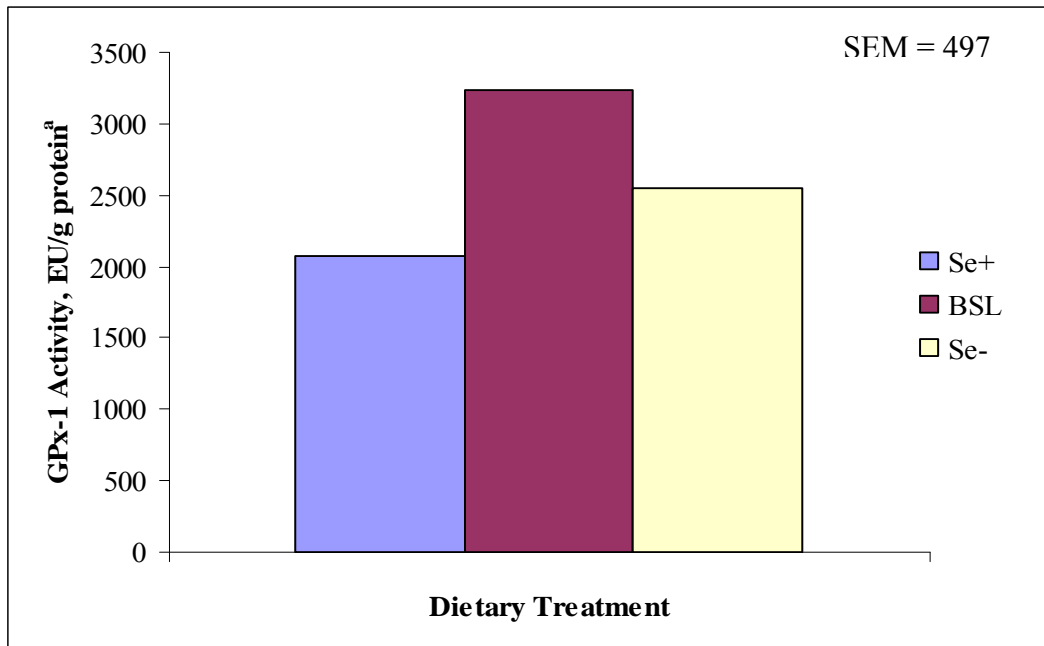
The trend observed in RBC-GPx activity occurred as expected, with Se adequate calves maintaining a relatively constant RBC-GPx activity while the RBC-GPx activity of Se-deficient calves decreased over time. The RBC-GPx activity for both treatment groups supported previous research in which animals maintained on a Se-deficient diet were reported to have significantly decreased RBC-GPx activity levels when compared to animals of the same species maintained on a Se adequate diet. Oh et al. (1976) reported that lambs maintained on a Se-deficient artificial milk diet had RBC-GPx activity levels that were significantly decreased ( $P < 0.01$ ) when compared to that of lambs maintained on a diet supplemented with 0.1 ppm Se. Weiss et al. (1996) reported that female rats maintained on a Se-deficient basal diet containing 0.007 ppm Se had an average RBC-GPx activity that was only 40% of the activity observed in rats maintained

on the basal diet supplemented with 0.1 ppm Se as sodium selenite. Rowntree et al. (2004) reported that pregnant beef cows either unsupplemented with Se or supplemented with a 20 mg Se drench had RBC-GPx activity levels of 14.94 EU/g Hb and 24.61 EU/g Hb, respectively, although these activities were actually somewhat lower than those found in our research using neonatal Holstein calves.

Liver GPx-1 activity on d 180 of age was not affected by dietary treatment ( $P= 0.581$ ). The average liver GPx-1 activities for Se adequate and Se-deficient groups were  $2079.33 \pm 943.20$  and  $2553.13 \pm 816.84$  EU/g protein, respectively, while that of the baseline calves was  $3241.25 \pm 943.2$  EU/g protein (Figure 3.9). The liver GPx-1 activity of both the Se adequate and baseline groups was a mean of the activities from three calves, while the activity of the Se-deficient group was the mean of four calves. A calf in the Se adequate group was removed from the analysis as it was determined to be an outlier.

These findings are not in agreement with any literature currently available. Oh et al. (1976) reported a significant increase ( $P < 0.01$ ) in the liver tissue GPx-1 activity of 8 wk old lambs fed a torula yeast-based artificial milk diet supplemented with either 0.05 or 0.1 ppm Se, versus lambs fed the same diet unsupplemented with Se. Likewise, Whanger and Butler (1988) reported that rats fed a Se-deficient basal diet containing 0.02 ppm Se had an average liver GPx-1 activity of  $8 \pm 4$  EU/g protein, representing 1.1% of the activity observed in rats maintained on the basal diet supplemented with 0.2 ppm Se as sodium selenite. Furthermore, Weiss et al. (1996). reported female rats maintained on a basal diet containing 0.007 ppm Se had an average liver GPx-1 activity that was only  $2 \pm 1\%$  of the activity observed in female rats maintained on the basal diet supplemented with 0.1 ppm Se as sodium selenite. It was considered that perhaps the liver GPx-1 activity response in larger mammals was not as profound as that observed in rats, but Lei et al. (1998) reported that pigs fed a diet containing 0.03 ppm Se had an average liver

GPx-1 activity of 12.5 EU/g protein, whereas pigs maintained on the basal diet supplemented with 0.3 ppm Se had an average liver GPx-1 activity of 210 EU/g protein, representing a 94% decrease in liver GPx-1 activity in pigs fed the Se-deficient diet.



<sup>a</sup> EU= enzyme unit; 1 EU is the amount of GPx activity required to oxidize 1  $\mu$ mol NADPH per min.

**Figure 3.9.** Mean liver GPx-1 activity of 21 d old baseline calves, and Se supplemented, and Se unsupplemented calves on d 180 of age.

Glutathione S-transferase (GST; EC 2.5.2.18) activity was considered as a possible explanation for the unexpected results obtained in this study. Glutathione S-transferase is a Se-independent glutathione peroxidase (Burk, 1983) associated with the reduction of certain organic hydroperoxides (Ullrey, 1987). These GST enzymes have been reported to be most active in tissues such as brain, liver, kidneys, adrenals, and testes, while tissues such as spleen, muscle, lungs, erythrocytes, thymus, and plasma have relatively little or no GST activity associated with them (Behne and Wolters, 1983). Research has shown that the use of organic hydroperoxides such as cumene hydroperoxide in the assay of GPx-1 activity in some tissues may result in

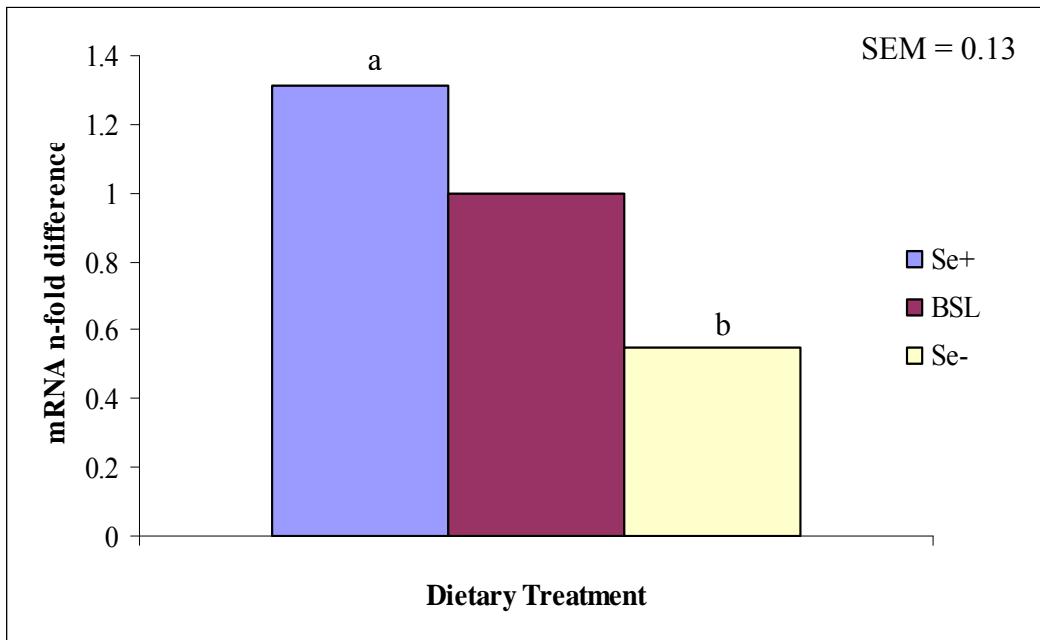
falsely elevated GPx-1 activity levels, especially in tissues of animals maintained on Se-deficient diets (Lawrence and Burk, 1976). In fact, Lei et al. (1998) reported that GST activity for pigs fed a Se-deficient basal diet containing 0.03 ppm Se or the basal diet supplemented with either 0.1 or 0.3 ppm Se as sodium selenite had liver GST activities of 513.4, 448.9, and 264.6 nmol S-2,4-dinitrophenylglutathione per min per mg protein, respectively. This finding supported previous research in which it was determined that GST activity was induced by Se deficiency (Christensen et al., 1994; Cheng et al., 1997). However, GST cannot catalyze the reduction of H<sub>2</sub>O<sub>2</sub>. For this reason, when liver GPx-1 activity assays were performed for this experiment, H<sub>2</sub>O<sub>2</sub> was used as the substrate in order to eliminate the accidental assay of GST and overestimation GPx-1 activity. Catalase, an iron-requiring peroxidase enzyme, could have contributed to the overestimation of GPx-1 activity in the liver samples from Se-deficient calves. However, two catalase inhibitors, sodium azide (NaN<sub>3</sub>) and cyanide contained in Drabkin's Reagent, were thus added to the reaction to inhibit the activity of this enzyme.

Most likely, the small sample size of calves euthanized for collection of liver samples played a part in both the unusually high standard error of the mean and the unexpected results observed regarding liver GPx-1 activity. In addition, calves were only sampled once, at trial termination, due to the large amount of tissue required for determination of liver Se concentration, liver GPx-1 activity, and liver GPx-1 mRNA relative transcript level analysis. We hypothesize that sampling of calves for liver samples at 28 d intervals, as was performed in the collection of blood and plasma, may be beneficial in obtaining a more realistic picture of liver GPx-1 activity over time. This might be accomplished in the future by performing liver biopsies for collection of liver tissue at pre-determined intervals in lieu of euthanizing calves.

### Liver GPx-1 mRNA Transcript Levels

Relative liver GPx-1 mRNA transcript level on d 180 of age was significantly higher ( $P < 0.05$ ) for the Se adequate group. On average, Se adequate calves had a relative mRNA level that was approximately 30% greater than the level of mRNA expression in baseline calves, while Se-deficient calves had approximately a 45% decrease in average relative mRNA level when compared to that of baseline calves. The difference in mRNA transcript level of the Se adequate treatment group versus the Se-deficient treatment group was considered to be significant. (Figure 3.10).

These observations are in agreement with previous research examining the effect of Se deficiency on relative liver GPx-1 transcript levels, although the extent to which the GPx-1 mRNA levels decreased varied among species. Weiss et al. (1996) reported that GPx-1 mRNA



<sup>a,b</sup> Different letters denote significant difference ( $P < 0.05$ ).

**Figure 3.10.** Relative GPx-1 mRNA transcript levels of baseline, Se supplemented, and Se unsupplemented calves expressed as n-fold differences.

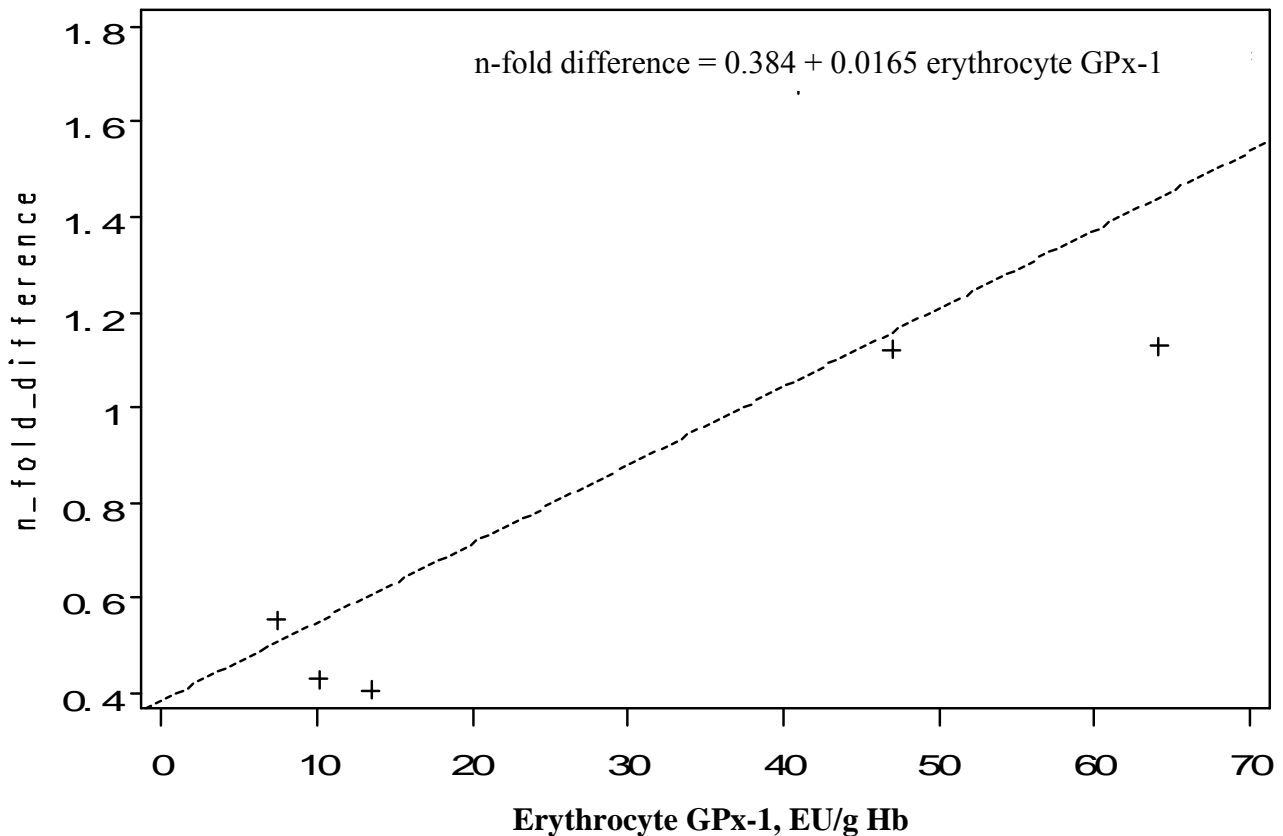
levels normalized to 18S rRNA of female rats fed a basal diet containing 0.007 ppm Se were  $17 \pm 4\%$  of those found in rats fed the basal diet supplemented with 0.1 ppm Se. Lei et al. (1998) determined that liver GPx-1 mRNA levels of pigs fed a Se-deficient basal diet containing 0.03 ppm Se were significantly lower ( $P < 0.05$ ) than that of pigs maintained on the basal diet supplemented with 0.1 or 0.3 ppm Se as sodium selenite. However, these findings are not in agreement with those of Hostetler et al. (2006), in which sows fed a Se-deficient diet containing 0.05 ppm were found to have no significant decrease ( $P > 0.05$ ) in liver GPx-1 mRNA level when compared to sows consuming a diet containing 0.39 ppm Se. Based on these results, calves in our study maintained on the Se-deficient diet may not have achieved severe Se deficiency. To our knowledge this is the first report of the effect of Se deficiency on relative liver GPx-1 mRNA transcript levels in the bovine.

Regression analyses were performed to determine the relationship between GPx-1 mRNA transcript level and both RBC-GPx and GPx-3 activity. There was a positive linear relationship between liver GPx-1 mRNA n-fold difference and RBC-GPx activity (Adjusted  $r^2 = 0.47$ ;  $r^2 = 0.58$ ; Figure 3.11). There was also a positive linear relationship between GPx-1 mRNA n-fold difference and GPx-3 activity (Adjusted  $r^2 = 0.76$ ;  $r^2 = 0.81$ ; Figure 3.12).

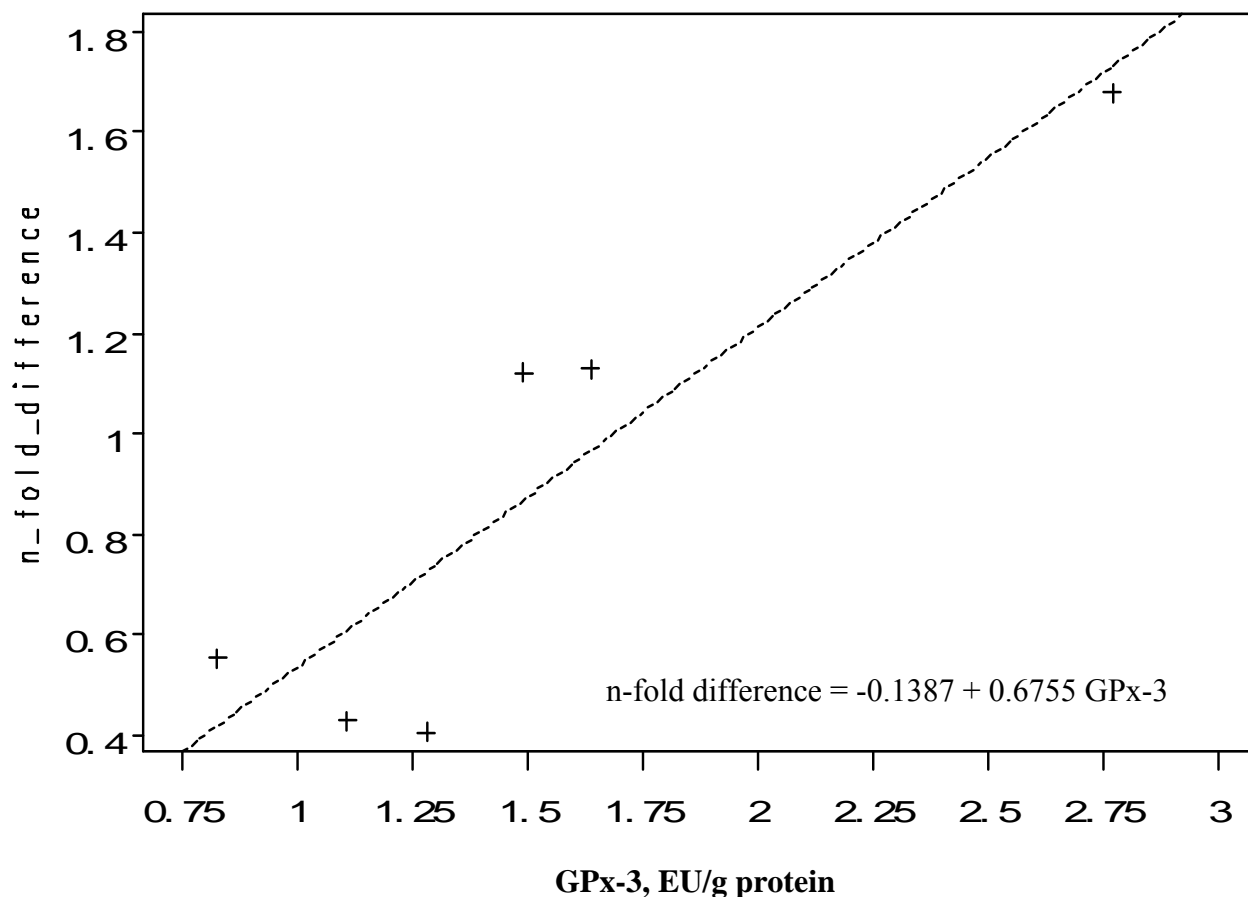
Correlation analyses were also performed to determine the extent of the relationship between the variables in each of the regression analyses. Erythrocyte GPx-1 activity and GPx-1 mRNA n-fold difference were moderately correlated, with a Pearson's Correlation Coefficient of 0.70. Interestingly, GPx-3 activity and GPx-1 mRNA n-fold difference were highly correlated, with a Pearson's Correlation Coefficient of 0.90.

The positive linear relationship observed between liver GPx-1 mRNA levels and both RBC-GPx and GPx-3 were expected, as GPx mRNA transcript level is expected to increase sigmoidally in response to Se supplementation (Weiss et al., 1996), while both RBC-GPx and

GPx-3 activities increase with increasing Se supplementation until reaching a specific plateau point (Weiss et al., 1996; Lei et al., 1998). However, the strength of both the linear relationship and the correlation coefficient for GPx-1 to GPx-1 mRNA levels was unexpected, as these were higher for GPx-3 than they were for RBC-GPx. It is currently unclear as to whether or not the strength of these relationships is truly significantly greater than that for the relationship between RBC-GPx and GPx-1 mRNA levels. It is possible that sampling calves for liver GPx-1 mRNA transcript level at only one time point, d 180 of age,



**Figure 3.11.** Regression analysis of relative GPx-1 mRNA n-fold difference and erythrocyte GPx-1 activity (Adjusted  $r^2=0.47$ ;  $r^2= 0.58$ ).



**Figure 3.12.** Regression analysis of relative GPx-1 mRNA n-fold difference and GPx-3 activity (Adj  $r^2= 0.76$ ;  $r^2= 0.81$ ).

only provided a partial picture of the ongoing relationship between RBC-GPx activity and subsequent mRNA levels. We hypothesize that liver biopsy and sampling of blood and plasma to obtain samples at consistent intervals may provide a clearer determination of the relationship between liver GPx-1 mRNA transcript levels and enzyme activities.

### Conclusions

The goal of this research was to establish a range of values for RBC-GPx and GPx-3 activities, as well as for liver Se concentration of neonatal and growing Holstein calves until approximately d 180 of age. These specifications are particular to our laboratory based on the type of research we wish to perform in the future; thus it was considered pertinent to establish reference values for our own use. Secondly, we investigated the effect of Se deficiency on

relative GPx-1 mRNA transcript levels in the 180 d old bovine calf. To our knowledge, there is currently no such research in publication.

The utilization of semi-purified and purified diets for the purpose of inducing Se deficiency is often very costly, especially in the feeding of large animals. It may be beneficial in the future to determine the minimal length of time required for the feeding of such diets to obtain Se deficiency, in order to minimize the amount of time animals must be maintained on such diets. The use of RBC-GPx or GPx-3 activity as an indicator of Se status in the growing Holstein calf may prove to be a valuable tool; however, determination of serial erythrocyte and plasma Se concentrations is necessary to truly establish the GPx activity level at which circulating Se levels may be considered deficient. In addition, determination of serial liver Se concentrations may prove equally beneficial, as liver Se concentration is generally considered the most accurate indicator of Se status.

The quantification of bovine GPx-1 mRNA in the liver of growing calves may also contribute to the current understanding of the effect of Se supplementation and deficiency on liver GPx-1 gene expression in the bovine. Considerable research on this subject has been performed in rats and, to a lesser extent, pigs, but very little information is available in for cattle. Understanding the effects of Se deficiency at the molecular level may provide valuable insight into the nature of selenium's role in health and immunity. However, our research is only a starting point; questions remain to be answered regarding the specific biology of Se in the ruminant animal. Important comparisons remain to be made between the biology of Se on the molecular level in the ruminant animals versus other large animal species. Trials with larger sample sizes utilizing serial sampling of liver and other tissues such as the tissues from the kidney may be beneficial in furthering our understanding concerning the role of Se in health on a molecular level.

There is still considerable information that remains to be learned concerning selenium as a micronutrient integral in oxidant status and the proper functioning of the immune system. Basic research is required to increase our understanding of antioxidants in immunity and health, both on the cellular level and with regards to the body as a whole.

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## **APPENDIX A: PROTOCOLS**

### **PLASMA AND ERYTHROCYTE COLLECTION PROTOCOL**

#### **Blood Collection**

1. Collect approximately 10 mL whole blood via jugular veinipuncture in 10 mL vacutainer tube (BD Vacutainer Systems, Franklin Lakes, NJ) containing sodium heparin to keep blood from clotting.
2. Mix blood and heparin together by gently inverting tube 3 or 4 times post-draw.
3. Store collected sample on ice until processing.

#### **Blood Processing**

1. Centrifuge vacutainer tubes for 15 min at 2,000 x g at 4° C.
2. After centrifugation, remove plasma layer by pipetting with a disposable pipet and store plasma in a 5 mL Falcon tube (BD Biosciences, San Jose, CA). Avoid removing white buffy layer from the surface of erythrocytes. Store plasma at -80° C until assay.
3. Remove white buffy layer from surface of erythrocyte layer and discard.
4. Wash erythrocytes by adding approximately 6 mL of ice cold phosphate buffered saline. Mix by gentle inversion and centrifuge at 2,000 x g at 4° C for 15 min.
5. Remove resulting supernatant and discard.
6. Repeat steps 5 and 6 two more times, for a total of three washings.
7. Following the last wash, pipet approximately 0.5 to 1.0 mL of erythrocytes into a 1.5 mL microcentrifuge tube. Freeze erythrocytes at -80° C until assay.

## **GLUTATHIONE PEROXIDASE ACTIVITY ASSAY PROTOCOL**

### **Pre-Assay Preparation**

#### **A. Assay Buffer (10x, 50 mM Tris-HCL, pH 7.6, 5 mM EDTA)**

1. Dilute 2 mL assay buffer concentrate, provided in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), with 18 mL of HPLC-grade water.
2. Store diluted buffer at 4° C for no longer than one month.

#### **B. Sample Buffer (10x, 50 mM Tris-HCL, pH 7.6, 5mM EDTA, 1 mg/mL BSA)**

1. Dilute 2 mL of sample buffer concentrate, provided in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), with 18 mL of HPLC-grade water. This buffer should be used to dilute the samples prior to assaying.
2. Store diluted buffer at 4° C for no longer than one month.

#### **C. Dilution of Bovine Glutathione Peroxidase**

1. Make two 10.5 µL aliquots of Bovine GPx, supplied in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), and store in a 0.5 mL microcentrifuge tube at -20° C.
2. Immediately prior to use, pipet 10 µL of the Bovine GPx to a 1.5 mL microcentrifuge tube and dilute with 490 µL Sample Buffer. Store on ice until assay. The diluted enzyme is stable for four hours on ice.

#### **D. Co-Substrate Mixture (lyophilized NADPH, glutathione, and glutathione reductase)**

1. Reconstitute each vial by adding 2ml of HPLC-grade water to each vial containing Co-Substrate Mixture and vortex well. The reconstituted agent should be kept at 25°C while assaying.

2. The reconstituted mixture should be stored at 4° C and is stable for 2 days.

Reconstituted mixture should not be frozen.

## **Sample Preparation**

### Plasma and Erythrocyte Lysate

1. Plasma stored at -80 °C must be thawed on ice prior to assay. No further dilutions are necessary.
2. Erythrocytes must be lysed by diluting 1:4 in ice cold HPLC-grade water.
3. Dilute erythrocytes 1:400 by adding 2 µL of lysed erythrocytes to 198 µL of ice cold sample buffer. Store dilutions at -80 °C until assay.
4. Thaw frozen erythrocyte 1:400 dilutions on ice for assay.

## **Performing the Assay**

### 1. Plate Set-up

- A. Background or non-enzymatic wells: add 120µl of assay buffer and 50µl of co-substrate mixture to three wells.
- B. Positive Control wells (bovine erythrocyte GPx): add 100µl of assay buffer, 50µl of co-substrate mixture, and 20µl of diluted GPx (control) to three wells.
- C. Sample wells: add 100µl of assay buffer, 50µl of co-substrate mixture, and 20µl of sample to three wells.

2. Allow plate to sit for 5 min to come to room temperature.
3. Initiate the reactions by adding 20µl of cumene hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the cumene hydroperoxide as quickly as possible.
4. Carefully shake the plate for a few s to mix.
5. Incubate plate at 25° C in plate reader.

6. Read the absorbance once every min for six at 340nm using a plate reader to obtain 7 absorbance reads.

## **GPX ASSAY KIT PROTOCOL FOR DETERMINATION OF LIVER GPX**

### **Pre-Assay Preparation**

#### **A. Assay Buffer (10x, 50 mM Tris-HCL, pH 7.6, 5 mM EDTA)**

1. Dilute 2 mL assay buffer concentrate, provided in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), with 18 mL of HPLC-grade water.
2. Store diluted buffer at 4° C for no longer than one month.

#### **B. Sample Buffer (10x, 50 mM Tris-HCL, pH 7.6, 5mM EDTA, 1 mg/mL BSA)**

1. Dilute 2 mL of sample buffer concentrate, provided in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), with 18 mL of HPLC-grade water. This buffer should be used to dilute the samples prior to assaying.
2. Store diluted buffer at 4° C for no longer than one month.

#### **C. Dilution of Bovine Glutathione Peroxidase**

1. Make two 10.5 µL aliquots of Bovine GPx, supplied in the Cayman Chemical GPx Activity Assay Kit (Cayman Chemical Corporation, Ann Arbor, MI), and store in a 0.5 mL microcentrifuge tube at -20° C.
2. Immediately prior to use, pipet 10 µL of the Bovine GPx to a 1.5 mL microcentrifuge tube and dilute with 490 µL Sample Buffer. Store on ice until assay. The diluted enzyme is stable for four hours on ice.

#### **D. Co-Substrate Mixture (lyophilized NADPH, glutathione, and glutathione reductase)**

1. Reconstitute each vial by adding 2ml of HPLC-grade water to each vial containing Co-Substrate Mixture and vortex well. The reconstituted agent should be kept at 25°C while assaying.

2. The reconstituted mixture should be stored at 4° C and is stable for 2 days.

Reconstituted mixture should not be frozen.

E. Double Strength Drabkin's Reagent (24 mM sodium bicarbonate, 1.6 mM potassium cyanide, 1.2 mM potassium ferricyanide)

1. Dissolve all solid reagents in distilled H<sub>2</sub>O (see Appendix B, Double Strength Drabkin's Reagent). Store at 25° C.

F. 0.01 M Sodium Azide

1. Dissolve solid reagents in 100 mL distilled H<sub>2</sub>O (see Appendix B, 0.01 M Sodium Azide).

G. 12 mM Hydrogen Peroxide (see Appendix B, 12 mM Hydrogen Peroxide)

## **Sample Preparation**

Tissue Homogenate

1. Prior to dissection, perfuse tissue with phosphate buffered saline. Snap freeze tissue in liquid nitrogen and store at -80° C until assay.
2. Grind the tissue into a fine powder using a mortar and pestle under liquid nitrogen. Do not allow tissue to thaw.
3. Weigh approximately 1 g tissue into a 14 mL Falcon tube ((BD Biosciences, San Jose, CA). Into the tube add 10mL cold buffer (50 mM Tris-HCL, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram of tissue.
4. Centrifuge at 10,000 x g for 15 min at 4°C.
5. Remove the supernatant for assay and pipet into another 14 mL Falcon tube (BD Biosciences, San Jose, CA). Make one 1.5 mL aliquot and freeze both at -80°C. The sample will be stable for at least 1 month.

## Performing the Assay

### 1. Plate Set-Up

A. Background or non-enzymatic wells: add 90 $\mu$ l of assay buffer, 50 $\mu$ l of co-substrate mixture, 20 $\mu$ l of Drabkin's Reagent, and 10 $\mu$ l sodium azide to each of three wells.

B. Positive Control wells (bovine erythrocyte GPx): add 70 $\mu$ l of assay buffer, 50 $\mu$ l of co-substrate mixture, 20 $\mu$ l of Drabkin's reagent, 10 $\mu$ l of sodium azide, and 20 $\mu$ l of diluted GPx (control) to three wells.

C. Sample wells: add 70 $\mu$ l of assay buffer, 50 $\mu$ l of co-substrate mixture, 20 $\mu$ l of Drabkin's reagent, 10 $\mu$ l of sodium azide, and **20 $\mu$ l** of sample to three wells.

2. Initiate the reactions by adding 20 $\mu$ l of 12 mM hydrogen peroxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the hydrogen peroxide as quickly as possible.

3. Allow plate to sit for 5 min to come to room temperature

4. Carefully shake the plate for a few s to mix.

5. Incubate the plate at 25° C in the plate reader for the duration of the assay.

6. Read the absorbance once every min at 340nm using a plate reader to obtain at least 5 time points.

## CALCULATION OF GLUTATHIONE PEROXIDASE ACTIVITY ASSAY RESULTS

1. Determine the change in absorbance ( $\Delta A_{340}$ ) per min by:

a. Plotting the absorbance values as a function to obtain the slope (rate) of the linear portion of the curve *–or–*

b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min} = A_{340} (\text{Time 2}) - A_{340} (\text{Time 1}) / \text{Time 2 (min)} - \text{Time 1 (min)}$$

2. Determine the rate of  $\Delta A_{340}/\text{min}$  for the background or non-enzymatic wells and subtract this rate from that of the sample wells.

3. Use the following formula to calculate the GPx activity. The reaction rate at 340nm can be determined using the NADPH extinction coefficient of  $0.00373 \mu\text{M}^{-1}$ . One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP per min at 25°C.

$$\text{GPx activity} = [(\Delta A_{340}/\text{min})/0.00373 \mu\text{M}^{-1}] \times (0.19\text{ml}/0.02\text{ml}) \times \text{sample dilution} = \text{nmol}/\text{min}/\text{ml}$$

## BRADFORD PROTEIN ASSAY PROTOCOL

### Preparation of Standard Curve with 2 mg/mL BSA

1. Prepare standards in 1.5 $\mu$ L microcentrifuge tubes. Prepare standard dilutions using 2 mg/mL BSA (Bio-Rad Laboratories, Inc., Hercules, CA) according to table A.1.:

**Table A.1.** Standard curve dilution preparation with 2 mg/mL BSA.

Tube #	Standard volume ( $\mu$ L)	Source of Standard	Diluent Volume ( $\mu$ L)	Final [Protein], ng/mL
1	70	2 mg/mL stock	0	2,000
2	75	2 mg/mL stock	25	1,500
3	70	2 mg/mL stock	70	1,000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8 (blank)	---	---	70	0

2. Pipette 20  $\mu$ L from each microcentrifuge tube into each of three clean, dry cuvettes (curve is constructed in triplicate).
3. Add 1mL Coomassie Blue dye (Bio-Rad Laboratories, Inc., Hercules, CA) to each cuvette.
4. Allow cuvettes to incubate for at least 5 min. Samples should not be incubated for longer than 1 hour at room temperature.
5. Use blank to zero the spectrophotometer. Select standard curve set-up procedure on the spectrophotometer. Enter # of standards, # or replicates per standard (3), and enter standard concentrations when prompted.
6. Print out results and save in spectrophotometer.

### Standard 1 mL Assay with Coomassie Brilliant Blue G-250

1. Remove 1x Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA ) dye

reagent from 4°C storage and allow it to warm to ambient temperature. Invert the 1x dye reagent a few times before use.

2. Prepare sample dilutions in glass-filtered water. Plasma samples require 1:50 dilution. Liver tissue hemolysate samples require 1:10 dilution.
3. Pipet 20  $\mu$ L of each unknown sample solution into separate clean cuvettes. Add 1 mL of 1x Coomassie Blue dye (Bio-Rad Laboratories, Inc., Hercules, CA) to each cuvette and invert.
4. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hour at room temperature.
5. The absorbance wavelength is 595nm. Before analyzing samples, zero the spectrophotometer with the blank sample. Blank sample consists of 20 $\mu$ L glass-filtered water and 1 mL Coomassie Brilliant Blue dye (Bio-Rad Laboratories, Inc., Hercules, CA).
6. Measure the absorbance of the unknown samples, using the pre-saved standard curve made prior to running the samples for comparison calculations.

## **QUANTICHROM™ HEMOGLOBIN ASSAY KIT PROTOCOL**

### 1. 96-Well plate set-up

#### A. Blank and standard wells.

Pipette 50  $\mu$ l water (for blank) and 50  $\mu$ l standard (for standard) into wells of a clear bottom 96-well plate. Transfer 200  $\mu$ l water into the blank and standard wells.

#### B. Sample wells.

Blood samples should be diluted 100-fold in distilled water (n=100).

Transfer 50  $\mu$ l samples into wells in triplicate per sample. Avoid bubble formation during the pipetting steps.

2. Add 200  $\mu$ l Reagent to sample wells and tap plate lightly to mix.

3. Incubate 5 min at room temperature. Read OD at 400 nm. Signal is stable for at least 2 hours.

## **LIVER mRNA ISOLATION PROTOCOL**

### **Pre-isolation Set-up**

1. Bring all buffers except Tris-HCl to room-temperature prior to use.
2. Resuspend Dynabeads Oligo(dT)<sub>25</sub> (DynaL Biotech, Inc., Lake Success, NY, USA) fully before use by gentle pipetting.
4. Pre-heat a water bath to 75°C.
5. Prepare individual aliquots of all buffers and reagents required for reaction prior to RNA isolation procedure.
6. Prepare Dynabeads Oligo(dT)<sub>25</sub> (DynaL Biotech, Inc., Lake Success, NY, USA) for isolation procedure.
  - a. Transfer 250µl of beads from the stock tube to a RNase-free 1.5ml microcentrifuge tube and place the tube on a magnet.
  - b. After 30 s (or when the suspension is clear) remove the supernatant.
  - c. Remove the vial from the magnet and wash the beads by resuspending in 250µl of fresh Lysis/Binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, 5 mM dithiothreitol).
  - d. When the sample lysate is ready for combination with the beads, place the tube on the magnet and remove the lysis/binding buffer after 30 s (or when the suspension is clear).

### **Preparation of Lysate from Liver Tissue**

1. Weigh and handle the animal tissue while frozen to avoid RNA degradation. Do not exceed the specified amount of tissue, as using too much tissue will reduce the mRNA yield and purity.
2. Treat clean, autoclaved mortar and pestle with RNase-Zap to remove trace RNases.

3. Freeze the microcentrifuge tube into which the ground tissue will be placed by placing it in dry ice. Weigh the frozen microcentrifuge tube, using it to zero the scale, and replace the tube in dry ice.
4. Pre-freeze the mortar and pestle in liquid nitrogen prior to adding tissue. Grind frozen tissue in liquid nitrogen. Work quickly.
5. Transfer the frozen powder to the microcentrifuge tube. Quickly add 1250 $\mu$ L Lysis/Binding Buffer to tube/sample and homogenize for 1 to 2 min until complete lysis is obtained. Rapid lysis in the Lysis/Binding Buffer is critical for obtaining undegraded mRNA.
6. Shear the DNA by forcing the lysate 3-5 times through a 21 gauge needle using a 1-2ml syringe. Use force.
7. Centrifuge the lysate for 30-60 s at approximately 10,000 x g to remove debris and reduce foaming after shearing.
8. Transfer the lysate to the tube containing the prepared Dynabeads.

### **RNA Isolation Procedure**

1. Mix the beads with the sample lysate and incubate with continuous mixing on roller mixer for 3-5 min at room temperature to allow the Poly-A tail of the mRNA to anneal to the oligo-dT on the beads.
2. Place the vial on the magnet for 2 min and remove the supernatant. Place supernatant into a clean RNase-free 1.5ml microcentrifuge tube and place on ice.
3. Wash the beads/mRNA complex two times with 2ml of Washing Buffer A ( 10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1mM EDTA, 0.1% lithium dodecylsulfate) at room temperature. Use the magnet to separate the beads from the solution between each washing step, removing the tube from the rack prior to adding washing buffer each

- time. Fully resuspend the Dynabead/mRNA complex at each washing by short, gentle vortexing or pipetting
4. Wash the beads/mRNA complex once with 1-1.5ml Washing Buffer B (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1 mM EDTA) at room temperature. Be sure to fully resuspend the beads in the washing buffer by gently vortexing or pipetting. Use the magnet to separate the beads from the solution.
  5. To elute the beads, add 25 $\mu$ l 10mM Tris-HCl (Elution Buffer), ensuring that the beads are completely resuspended in solution by gentle pipetting, and incubate at 65-80°C for 2 min. Place the tube immediately on the magnet and transfer the supernatant containing the mRNA to a new RNase-free tube. Place the mRNA immediately on ice.

## **cDNA SYNTHESIS PROTOCOL**

1. Mix 4  $\mu\text{L}$  of iScript reaction mix, 1  $\mu\text{L}$  of reverse transcriptase, and 5  $\mu\text{L}$  of autoclaved water.
2. Make master mixes when possible.
3. Add 10  $\mu\text{L}$  of mRNA sample.
4. Extra mix should be prepared for the no mRNA template negative control.
5. Total volume mix should be 20  $\mu\text{L}$ .
6. Place the mix in the thermocycler.
7. Run the thermocycler at 25°C for 5 min, 42 °C for 30 min, denaturation at 85 °C for 5 min, and a final hold at 4 °C.

## REVERSE TRANSCRIPTASE PCR PROTOCOL

1. Mix 5  $\mu\text{L}$  of 10X PCR buffer, 1  $\mu\text{L}$  dNTP mix (Appendix B), 2  $\mu\text{L}$  Jump Start™ REDTaq™ DNA Polymerase (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 39  $\mu\text{L}$  water. Add 1  $\mu\text{L}$  of each 20 pmol primer. Make a larger volume for multiple samples when appropriate (Master Mix; Appendix B, rtMM).
2. Add 1  $\mu\text{L}$  of the cDNA sample to each of the master mixes containing the 20 pmol primers.
3. Prepare enough of each master mix, both the mix without the 20 pmol primers and the mix with the primers added, for the negative control, into which no cDNA will be added.
4. Place the sample into the thermocycler.
5. Run one cycle of 1 min at 95°C; 35 cycles of PCR (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s); followed by 72 °C for 4 min with a final hold temperature of 4 °C.

## QUANTITATIVE PCR PROTOCOL

1. Mix 12.5  $\mu\text{L}$  of iQ SYBR<sup>™</sup> Green 2X Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1  $\mu\text{L}$  of 20 pmol Primer 1, 1 of 20 pmol  $\mu\text{L}$  of Primer 2, and 9.5  $\mu\text{L}$  autoclaved water for each well.
2. A master mix should be formulated for each gene of interest (Master Mix; Appendix B, bGPxMM or PaMM).
3. Prepare enough master mix to run both the calibrator and negative controls containing no cDNA for each gene of interest.
4. Pipette 96  $\mu\text{L}$  of bGPxMM into one 0.5 mL microcentrifuge tube for each sample (calf)/calibrator/NT control.
5. Add 4  $\mu\text{L}$  cDNA/calibrator/H<sub>2</sub>O for NT control to the corresponding microcentrifuge tube containing 96  $\mu\text{L}$  bGPxMM.
6. Pipette 96  $\mu\text{L}$  of PaMM into one 0.5 mL microcentrifuge tube for each sample (calf)/calibrator/H<sub>2</sub>O for NT control.
7. Add 4  $\mu\text{L}$  cDNA/calibrator/H<sub>2</sub>O for NT control to the corresponding microcentrifuge tube containing 96  $\mu\text{L}$  PaMM.
8. Pipette 25  $\mu\text{L}$  from each sample for each gene of interest into each of three wells.
9. Place the 96-well plate into the thermocycler.
10. Run one cycle of 3 min at 95°C; 40 cycles of PCR (95 °C for 10 s and 56 °C for 45 s); a melting curve consisting of 95 °C for 1 min followed by 55 °C for 1 min, a step cycle with 0 repeats starting at 55 °C for 10 s with a +0.5 °C/s transition rate; and a final hold at 4 °C.

## GENE EXPRESSION QUANTIFICATION PROTOCOL

1. Use the method described by Pfaffl (2001).
2. Use the combined cDNA from the baseline calves (Materials and Methods) as the calibrator for the target genes. The same calibrator should be used throughout all the experiments and plates.
3. Use Poly-A polymerase as the endogenous control gene.
4. Obtain efficiencies by performing a 10-fold dilution standard curve on all genes of interest, both the reference and target genes, prior to analysis.
5. Use the signal of the reference gene Poly-A to normalize the target gene signals of each sample by subtracting the average  $C_T$  value of Poly-A from that of the target gene.
6. Calibrate the  $\Delta C_T$  for gene transcription against the sample used as the calibrator. Calculate both a  $\Delta C_{T,T}$  for the target gene and a  $\Delta C_{T,R}$  for the reference gene.
  - a. Calculate the  $\Delta C_{T,T}$  by subtracting the  $C_T$  value for the target gene in the calibrator from the  $C_T$  value for the target gene in each sample.
  - b. Calculate the  $\Delta C_{T,R}$  by subtracting the  $C_T$  value for the reference gene in the calibrator from that of the reference gene in each sample.
7. Calculate the relative linear amount of target molecules relative to the calibrator by using the following equation:

$$\text{n-fold difference} = \text{Efficiency Target Gene}^{\Delta C_{T,T}} / \text{Efficiency Reference Gene}^{\Delta C_{T,R}}$$

**APPENDIX B: BUFFER AND STOCK PREPARATIONS**

**PHOSPHATE BUFFERED SALINE <sup>a</sup>**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Amount</b>
Sodium Chloride	S271-500	Fischer Scientific	80.0 g
Potassium Chloride	PX1405-CB607	Matheson Coleman and Bell	2.0 g
Sodium Phosphate	7917-1	Mallinckrodt	11.5 g
Potassium Phosphate	P0662-500G	Sigma	2.0 g
ddH <sub>2</sub> O	-	-	1000 mL

<sup>a</sup> 10X concentrate. Store at 4°C. Dilute to 1:10 in ddH<sub>2</sub>O for use in washing cells and perfusing tissue.

**TISSUE HOMOGENATION BUFFER <sup>a</sup>**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Final mM</b>	<b>mL, mg / 250 mL</b>
1 M Tris-HCl, pH 7.5	15567-027	Invitrogen	50	12.5
EDTA	JT8993-1	JT Baker	5	465.3
Dithiothreitol	D-1532	Invitrogen	1	38.5
ddH <sub>2</sub> O	-	-	-	237.5

<sup>a</sup> DTT has short half-life. Use immediately; do not store!

**DOUBLE STRENGTH DRABKIN'S REAGENT**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Final mM</b>	<b>mL, mg / 100 mL</b>
Sodium Bicarbonate	S6014-25G	Sigma-Aldrich	24	200
Potassium Cyanide	60178-25G	Sigma Aldrich	1.6	10.4
Potassium Ferricyanide	244023-5G	Sigma-Aldrich	1.2	39.6
dd H <sub>2</sub> O	-	-	-	100

### 0.01 M SODIUM AZIDE SOLUTION

Component	Product No.	Company	mL, mg / 100 mL
Sodium Azide	S8032-25G	Sigma-Aldrich	65
ddH <sub>2</sub> O	-	-	100

### 12 mM HYDROGEN PEROXIDE<sup>a,b</sup>

Component	Product No.	Company	mL/100 mL
30% H <sub>2</sub> O <sub>2</sub> , w/w	216763-100ml	Sigma-Aldrich	0.124
ddH <sub>2</sub> O	-	-	99.876

<sup>a</sup> Store capped in dark vial at 4° C.

<sup>b</sup> Decant small amount into separate vial for each assay.

### iSCRIPT REVERSE TRANSCRIPTASE cDNA SYNTHESIS MIX

Component	Product No.	Company	Amount
iScript Reaction Mix	170-8891	BioRad	4 µL
Reverse Transcriptase	170-8891	BioRad	1 µL
mRNA	-	-	10 µL
Autoclaved H <sub>2</sub> O	-	-	5 µL

### dNTP MIX <sup>a</sup>

Component	Product No.	Company	Amount
dATP	1-969-064	Roche	10 µL
dCTP	1-969-064	Roche	10 µL
dGTP	1-969-064	Roche	10 µL
dTTP	1-969-064	Roche	10 µL
Autoclaved H <sub>2</sub> O	-	-	60 µL

<sup>a</sup> Store at -20°C for 2 to 3 months

**REVERSE TRANSCRIPTION PCR MASTER MIX (rtMM)**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Amount (Per Sample)</b>
Autoclaved H <sub>2</sub> O	-	-	39 µL
10X PCR Buffer	D-8187	Sigma	5 µL
dNTP Mix	-	-	1 µL
Primer 1	-	Invitrogen	1 µL
Primer 2	-	Invitrogen	1 µL
Jump Start Taq Polymerase	D-8187	Sigma	2 µL

**REVERSE TRANSCRIPTION PCR MIX**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Amount (Per Sample)</b>
rtMM	-	-	49 µL
cDNA	-	-	1 µL

**QUANTITATIVE PCR REACTION MIX FOR bGPx**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Amount (Per Well)</b>
SYBR Green 2X Supermix	170-8882	BioRad	12.5 µL
bGPx Primer 1	-	Invitrogen	1 µL
bGPx Primer 2	-	Invitrogen	1 µL
Autoclaved H <sub>2</sub> O	-	-	9.5 µL

**QUANTITATIVE PCR REACTION MIX FOR POLY-A**

<b>Component</b>	<b>Product No.</b>	<b>Company</b>	<b>Amount (Per Well)</b>
SYBR Green 2X Reaction Mix	170-8882	BioRad	12.5 µL
Poly-A Primer 1	-	Invitrogen	1 µL
Poly-A Primer 2	-	Invitrogen	1 µL
Autoclaved H <sub>2</sub> O	-	-	9.5 µL

### QUANTITATIVE PCR MASTER MIX FOR bGPx (bGPxMM)

Component	Product No.	Company	Amount (Per Gene) <sup>a</sup>
SYBR Green 2X Reaction Mix	170-8882	BioRad	512.5 µL
bGPx Primer 1	-	Invitrogen	41 µL
bGPx Primer 2	-	Invitrogen	41 µL
Autoclaved H <sub>2</sub> O	-	-	389.5 µL

<sup>a</sup> Analyzing 41 reactions (3 wells per calf/calibrator/NT + 1 per calf/calibrator/NT)

**Note:** Add an additional reaction to each calf/calibrator/NT to ensure enough in case of pipetting error.

### QUANTITATIVE PCR MASTER MIX FOR POLY-A (PaMM)

Component	Product No.	Company	Amount (Per Gene) <sup>a</sup>
SYBR Green 2X Reaction Mix	170-8882	BioRad	512.5 µL
Poly-A Primer 1	-	Invitrogen	41 µL
Poly-A Primer 2	-	Invitrogen	41 µL
Autoclaved H <sub>2</sub> O	-	-	389.5 µL

<sup>a</sup> Analyzing 41 reactions (3 wells per calf/calibrator/NT + 1 per calf/calibrator/NT)

**Note:** Add an additional reaction to each calf/calibrator/NT to ensure enough in case of pipetting error.

### QUANTITATIVE PCR MASTER MIX FOR EACH SAMPLE/CALIBRATOR/NT (MMsamp)

Component	Product No.	Company	Amount (Per Sample)
bGPxMM or PaMM	-	-	96 µL
cDNA <sup>a</sup> /calibrator/H <sub>2</sub> O for NT control	-	-	4 µL

<sup>a</sup> 1:10 dilution of cDNA was used for all samples and calibrator.

## VITA

Genevieve Elizabeth Lum was the first child born to Donald Kelly and Genevieve Schoeffler Lum of Clinton, Louisiana. Genevieve has two younger siblings, Lauren Meagan and Daniel Jacob, both attending Louisiana State University. Lauren is currently pursuing a degree in finance and Daniel a degree in physics. Genevieve was raised in Clinton, Louisiana, until moving to Baton Rouge, Louisiana, to attend high school. She attended high school at Christian Life Academy in Baton Rouge, Louisiana, where Genevieve graduated valedictorian of her high school class.

After graduating high school in May 2001, Genevieve attended Louisiana State University, where she obtained her Bachelor of Science Degree in animal, dairy, and poultry Sciences in December of 2005. In the spring of 2005, Genevieve took part in undergraduate research under the supervision of Dr. John Chandler at the D.E. Patrick Dairy Herd Improvement Center in Baton Rouge, Louisiana.

She entered graduate school in January of 2006 under the direction of Dr. Jason Rowntree. She is now a candidate for the degree of Master of Science in the School of Animal Sciences at Louisiana State University in Baton Rouge, Louisiana. After graduation, Genevieve plans to pursue a doctoral degree in the School of Animal Sciences at Louisiana State University.