Abstract

Rivera, Rafael Edgardo. The Effect of Selenium on Heat Shock Protein 70 Expression in Turkey Embryos. (Under the direction of Vern L. Christensen.)

The optimum incubation environment to obtain the best poult quality has always been an important issue in the turkey industry. Heat shock protein 70 (hsp70) has been associated with the acquisition of thermal tolerance and tolerance to other stressors in avian species. The hsp70 family of proteins functions as a molecular chaperone, assisting in protein refolding after stress-induced denaturation. Selenium is an essential trace mineral and must be incorporated into the enzyme glutathione peroxidase (GPx). GPx functions in the GSH/GSSG antioxidant system and protects cells from damage due to oxidative stress. This study was conducted to examine if a potential relationship exists between dietary supplementation of selenium to turkey hens and the expression of hsp70 in heat-stressed turkey embryos. A total of 52 Nicholas turkey breeder hens were fed either a breeder diet with no supplemental selenium (Se) or one supplemented with 0.3ppm organic Se in yeast (Sel-Plex®, Alltech Inc., Nicholasville, KY). The experimental diets were fed to hens for a minimum of two weeks before the eggs were collected. Fertilized eggs were incubated at 37.5°C for 21 days, and the embryos then were heat stressed at 40°C for 2 hr. Liver samples were collected immediately after the termination of the heating episode. The liver samples were analyzed for hsp70 using an immunoblotting procedure. Se activity was assessed by determination of GPx activity. In Experiment 1, there were no significant differences ($P>0.05$) between treatment effects on hsp70 expression or GPx activity in embryonic liver samples.

A second experiment was conducted using commercial breeder flocks. A total of 100 eggs were collected from a non-Se-supplemented flock and 90 eggs were collected from a flock, fed 0.3 ppm of organic selenium in yeast (Sel-Plex®, Alltech Inc., Nicholasville, KY). The eggs were incubated for 21 days at 37.5°C. At day 22, half of the eggs were stressed at 40°C, and the other half remained at normal incubation temperature. The stressed eggs were returned to normal incubator temperature and allowed to recover for 3h. Livers were collected and analyzed for hsp70 and GPx. The

results of experiment 2 were compared with the results from experiment 1. Before stress, hsp70 concentrations were low while GPx activity was at a maximal level. During stress (Experiment 1), temperature caused a non-significant ($P>0.05$) induction of hsp70 and a significant ($P<0.05$) decrease of GPx with no significant differences between Se treatments; however, after stress recovery hsp70 concentrations were significantly ($P<0.05$) higher in the non-Se-supplemented groups while the Se-supplemented groups were near pre-stress concentrations. GPx activity in the non-Se-supplemented groups did not recover after stress but Se-supplemented groups were increasing significantly ($P<0.05$) toward the control non-stress activity.

Hsp70 functions are energy dependent. The hsp70 functions protect newly synthesized proteins during and after heat stress. When hsp70 is induced as a result of cellular exposure to a stressor, the cell will decrease normal protein production and divert its energy resources toward mechanisms of survival (hsp70 induction). Se supplementation helped to increase GPx activity before stress. During stress there was decreased GPx activity, which resulted in the induction of hsp70. After stress, Se supplementation allowed for increase in GPx activity, facilitating the reduction of oxidative stress products. Thus, cells from embryos from Se-fed dams did not require long-term protection provided by hsp70. Therefore, embryos with adequate Se from the dam can resume normal developmental functions more rapidly than embryos from dams with inadequate selenium nutrition.

THE EFFECT OF SELENIUM ON HEAT SHOCK PROTEIN 70 EXPRESSION IN TURKEY EMBRYOS.

by
RAFAEL E. RIVERA

A thesis submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Master of Science

POULTRY SCIENCE

Raleigh

2004

APPROVED BY:

_____ _____

_____ _____
Chair of Advisory Committee

DEDICATION

To my wife

Brenda L. Andújar Martínez

I LOVE YOU WITH ALL MY HEART

BIOGRAPHY

Rafael E. Rivera was born in Río Piedras, Puerto Rico from the marriage of Edgardo R. Rivera and Doris A. Betancourt. He was raised in Mayagüez, Puerto Rico where he graduated from the Western Adventist Academy in the summer of 1997. He graduated from the University of Puerto Rico at Mayagüez in 2001 with a Bachelor of Science degree, majoring in Animal Science. In August, 2001 he was admitted to the Graduate School at North Carolina State University to obtain a Master of Science degree in Poultry Science, under the direction of Dr. Vern L. Christensen, PhD. In July 19, 2003 he married Ms. Brenda L. Andújar Martínez.

ACKNOWLEDGEMENTS

The author would like to extend his appreciation to Dr. Vern Christensen for giving him the opportunity to study under his guidance. The author would like to thank especially José Bruno Barcena for his assistance, advice in carrying out the study, and his friendship.

The author would like to thank Dr. Frank Edens for challenging me and helping me to develop critical thinking in my work. The author would like to thank Debbie Ort and Sue Mann for their advice and help through my time here. Special Gratitude is given to Dr. Mike Wineland and Dr. Jesse Grimes for their review of this study, and Pam Jenkins for her help in the statistical analysis of the data.

The author would like to thank his family for inspiration. The author would like to thank his fellow graduate students, especially Kymberly Gowdy, Anael Santos, Fernanda Santos, Hugo Romero Sánchez, Sigfredo Burgos, Manzoor Cheema , Renee Plunske, Ondula Foye, and Panthong Singboottra for their advice and unconditional friendship.

The author would like to express his most special gratitude to his wife Brenda. Without her love and sacrifice this study could not have been completed. Finally and most important, the author would like to thank Our Lord Jesus Christ for teaching him the way of life.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

Literature Review

I. Introduction

Fast growing broilers and turkeys can suffer from metabolism-related diseases. However, the mechanism, the influence of different factors, and the optimum level of the conditions involved are not well understood (Meijerhof and Albers, 1998). Optimum incubation temperature for embryos of poultry species is typically 37.5°C, and small deviations from this optimum can have a significant adverse impact on hatchability and length of the incubation period (Wilson, 1991). Temperature is the most pervasive environmental factor that influences livestock and poultry (Al-Saffar and Rose, 2002). During the first day of life, a chick still reacts as a poikilotherm, and as a consequence, a lower ambient temperature leads to a drop in body temperature, depending on the isolation abilities and size of the chick (Weytjens et al., 1999). High temperature has a negative effect on broiler production efficiency. During heat stress, behavioral, physiological, hormonal, and molecular changes occur (Moraes et al., 2003).

From the moment of fertilization of the ovum, embryogenesis starts, and embryo development is affected by its environment (Decuypere and Michels, 1992). Although the rate of embryo growth appears to be narrowly regulated within a species, embryo development varies with respect to the age of breeders, duration and temperature of pre-incubation of egg storage, and incubation temperature (Yalcin and Siegel, 2003). In the incubation process of eggs (chicken, turkey, and duck), development of the living organism is mainly influenced by the physical microenvironment around the egg (Van Brecht et al., 2003). Birds exposed to high ambient temperatures divert part of their production energy to maintain thermal homeostasis, and manifest physiological responses

such as panting, decrease in feed intake and growth has been reported (Givisiez et al., 2001). Embryogenesis comprises a series of temporally and spatially organized events in gene expression and cellular signaling processes in order to provide successful development under adverse changes in the microenvironment (Gabriel et al., 2002). Molecular mechanisms responsible for improved thermotolerance have not been fully elucidated (Givisiez et al., 2001).

It is known that heat stress and other kinds of stressors (e.g. anoxia, and heavy metal ions) rapidly induce the synthesis of heat shock proteins (hsp), which, in a normal environment, are present at low levels (Givisiez et al., 2001). Also many hsps are expressed as an emergency response to diverse stressor agents, and that increased synthesis of these proteins seems to be involved in protection of stressed cells and organisms (Gabriel et al., 2002). There are other factors that might influence the stress response, such as selenium. Selenium (Se) is a required nutrient in the diets of poultry (Paton et al., 2002a). The eggs of avian species must support life independently for the period of incubation. As a result birds must incorporate all the necessary nutrients to support the development of the embryo into the egg before it is laid (Paton et al., 2002a). Incorporation of essential trace elements into the diets of all animals is required for maintenance of health, growth and myriad biochemical-physiological functions. Among those essential trace elements is Se (Edens, 2002). Se is required for the functioning of the enzyme glutathione peroxidase (GPx), which has a major role in antioxidant metabolism (Paton et al., 2002b). The main focus on this review is to mention physiological, molecular and management practices that might affect embryo management of temperature stress.

II. Temperature effects on embryos

A. Effects of incubation temperature on embryo heat management and development.

During incubation, the environmental conditions dictate the metabolism of the embryo (Meijerhof and Albers, 1998). This influences the hatching results but also can have an influence on the chick. Turkey eggs are normally incubated at 37.5°C, and increases of 1°C or more above the optimum have been shown to significantly reduce hatching success (French, 1997b). Growth efficiency of the embryo is affected by temperature (Wilson, 1991). Wilson (1991) showed that the optimum incubation temperature is between 37 and 38°C, with variation due to species, embryonic age, humidity and other factors. Wilson (1991) also stated that hyper- and hypothermic effects depend largely upon the degree of temperature, duration of exposure and embryonic age.

Much of the early incubation research involved exposing developing embryos to temperatures above that considered optimum (Wilson, 1991). The adverse effect of high temperature on embryo survival increases with increased temperature, increased exposure time, and age (Wilson, 1991). Susceptibility of embryos to heat stress is inversely related to heat production and oxygen consumption. (Wilson, 1991; Ande and Wilson, 1981). Ande and Wilson (1991) showed in dead embryos, death occurred soon after heat stressing, especially in 7-day and 19-day embryos. Days 7 and 19 increased mortality, thus, it was inferred that embryos at these stages could be sensitive to all types of stress. Ande and Wilson (1981) stated that this could be related to the chorio-allantoic membrane susceptibility to heat stress during this period. Turkey eggs incubated at

38.5°C had poor embryo survival (French, 1994). Over heated embryos had increased mortality between 15-20 days and 23-26 days of incubation (French, 1994). The greater sensitivity of embryos to high temperatures at the end of incubation may be due to increasing embryo metabolic heat production. French (1997b) stated that the time required for chickens to die from 48°C decreased when embryos aged 12 and 20 d of incubation. Measurements of internal egg temperatures showed that embryos at all ages died after internal egg temperatures reached 46-47°C, and the time taken to die was reduced with age because the initial temperature was increasing with age due to greater heat production (French, 1997b).

Disproportionate development, circulation disruption, abnormalities and reduced growth are common consequences of high or low temperatures (Wilson, 1991). Wilson (1991) also stated that as hyperthermia increases and growth decreases or stops, the incidence of malformation increases. The growth of embryonic liver is affected by sub-optimal incubation temperature (Romanoff et al., 1938). High incubation temperature resulted in the highest mortality levels due to ascites (Meijerhof and Albers, 1998). It seems that the extra availability of oxygen supplied more stress to the heart. In general, the temperature profile seemed to have more influence on the occurrence of ascites, while the air exchange in the air cell had more influence on the heart quality (Meijerhof and Albers, 1998). French (1994) found that overheated turkey embryos had a high incidence of subcutaneous hemorrhage, chorio-allantoic hemorrhage, opaque eye disc, swollen down pumule, edematous head, ruptured yolk sac, excess albumen, embryos upside down, and embryos with their head between the legs after 24 days.

Yalcin and Siegel (2003) showed that high temperatures had an effect on growth, especially showing differences in growth on tissues. It showed that temperatures affected tissue growth at different stages of incubation. The study showed asymmetries in skeletal traits early in incubation and tended to decrease toward hatching, showing growth compensation late in incubation (Yalcin and Siegel, 2003). Heat also had an effect showing short face length and low lung weight (Yalcin and Siegel, 2003). Chicks that hatched following heat stress tended to be weaker and there was an increased incidence of culls as the severity of heat stress increased. There was also a high incidence of clubbed wiry down and exhibited unsteady gait (Wilson, 1991).

Heat stress has an effect on general metabolism as well. An increase in environmental temperature can cause metabolizable energy to be diverted from production and growth to functions in the maintenance of homeothermy (Moraes et al., 2003). High temperatures reduces thyroid activity and subsequently, metabolic rate, oxygen consumption, feed intake, and growth rate (Moraes et al., 2003). Christensen et al., (2002) mentioned that the embryonic thyroid plays a major role in maturation of vital tissues during the final stages of in ovo life. Thyroid functions affect turkey embryonic hatching times as well as survival rates (Christensen et al., 2002). This will affect the maturity of the embryo at hatching and the amount of care needed after hatch (Christensen et al., 2002). Heat exposure lowered the respiratory quotient, reflecting an increase in fatty acid oxidation or gluconeogenesis to meet energy requirements (Moraes et al., 2003). During stress there is decrease on CO_2 production and an increase in O_2 consumption. That represents an increase in oxidation of fatty acids, conversion of

metabolites having a low O_2 content, such as protein, into metabolites having a high O_2 content such as carbohydrates (Moraes et al., 2003).

Romanoff et al. (1938) mentioned that an incubation temperature at 38.5°C initially promoted a high rate of body growth, but at 16d this had slowed to match that observed for embryos incubated at 37.5°C. Presumably slower growing individuals that resemble those at 37.5°C are able to survive prolonged exposure to high temperatures (Romanoff et al., 1938). After 21 days of development there is a reduction in the rate of growth of the body, liver, and heart. This coincides in a period where there is peak mortality in turkey embryos incubated at high temperatures (French, 1994). Decuypere and Michels (1992) mentioned that line differences could also be a factor in determining heat resistance of embryos. They showed that embryos from broiler hens are much more sensitive to temperature range, than embryos from layer strains. It has also been suggested that tolerance of variation in the physical environment may be less wide for domesticated species than for wild birds (Decuypere and Michels, 1992).

Cooling of embryos and its effects on developing embryos has been described in terms of temperature zones. Disproportionate development usually occurs in the temperature range between the physiological zeros, (27°C) to the lower side of the normal range (35°C) (Wilson, 1991). It appears that tissues may have different physiological zeros, thus resulting in uneven or unsynchronized growth (Wilson, 1991). Yalcin and Siegel (2003) showed that relative asymmetries were higher in lungs especially in embryos incubated at low temperatures. Also skeletal traits showed development at different rates, and showed high relative asymmetries, but they converged for a developmental stability prior to hatch (Yalcin and Siegel, 2003). Apparently there is

growth compensation during recovery of stress. Hatchling weight is higher for eggs exposed to low temperatures during incubation, perhaps due to lower moisture loss or the loss of weak and potentially smaller birds due to the cold stress (Wilson, 1991). French (1994) showed that turkey eggs incubated at 36.5°C had decreased embryonic survival than those incubated at 37.5°C due to increased mortality on days 26 and 27 of incubation and a high incidence of late hatching poults.

A lower ambient temperature leads to a drop in body temperature, depending on the isolation abilities and size of the chick (Weytjens et al., 1999). In contrast to older embryos a decrease in body temperatures due to low incubation temperature usually causes a decrease in net heat production (Nichelmann et al., 2001). Nichelmann and Tzschentke (1999) mentioned that factors that influence this result are species, age, ambient temperature, and duration of cooling. Generally altricial birds have no endothermic response during the prenatal period. It has been suspected that the wedge-tailed shearwater (*Puffinus pacificus*) embryo cannot sustain significant levels of cold induced stress (Mathiu et al., 1992). The reaction of the same species but different ages may be different, because enzyme activity, oxygen uptake, the development of the cardiovascular system, the function of the central nervous system and some other processes may differ with age (Nichelmann and Tzschentke, 1999). Surai (1999) showed that there is an increase in several antioxidant enzymes during chicken embryo development. The study indicates that different tissues display distinct development strategies with regard to the acquisition of antioxidant capacity (Surai, 1999). Evidence for a variety of birds indicates that the rate of diffusion of oxygen through the shell may

limit embryonic growth, oxygen uptake, and oxygenation of the blood, especially just before piping (Mathiu et al., 1992).

An endothermic reaction may only occur when the perception of the cold stimulus is possible. The activity of internal thermosensors decreases with the perception of the cold stimulus is possible. The activity of internal thermosensors decreases with decreasing local temperature, and at a threshold temperatures reception ceases (Nichelmann and Tzschentke, 1999). Generally, the increased measured HP in endothermic animals at temperatures below the thermoneutral temperature is the result of two different processes: the thermoregulatory neural network and the energy metabolism following the van't Hoff rule (Nichelmann and Tzschentke, 1999). The role of Q_{10} is also important in the interpretation of thermoregulatory mechanisms. Q_{10} is defined as the rate of a physiological process at a particular temperature to the rate at a temperature 10°C lower, when the logarithm of the rate is an approximately linear function of temperature (Nichelmann et al., 1998). Usually a Q_{10} above 2 is a signal of no endothermy; below 2 is a signal of endothermy. Nichelmann and Tzschentke (1999) showed that some embryos are able to increase their HP slightly and decrease the Q_{10} to values below 1.0 immediately before or after external pipping while Ta decreases. In contrast to older birds, in avian embryos a decrease in body temperature due to low temperature usually causes a decrease in net HP, but the decrease is more moderate than would be expected by the van't Hoff rule (Nichelmann et al., 1998). Conditions of HP at low ambient temperature improve with a higher diffusion of O_2 during internal and external pipping (Mathiu et al., 1992; Nichelmann and Tzschentke, 1999; Nichelmann et al., 1998). Avian embryos are poikilothermic because an inability to regulate body temperature during high

and low incubations. Because of the low efficiency of thermoregulatory effector systems in avian embryos, it could be argued that thermoregulatory mechanisms are not necessary in avian embryos, given that (1) the embryos are kept warm by the incubating parent in most avian species, for most of the time, and (2) thermal tolerance of the embryos protects them to some extent from overheating and cooling (Nichelmann and Tzschentke, 1999). These mechanisms of tolerance might be the cause of genetic expression and enzymatic activity. The development of these systems starts before the egg is laid so breeder management is important in the success of these embryos to resist temperature changes during incubation.

B. Effects of breeder management on embryo stress management.

From the moment of fertilization of the ovum and the initiation of embryogenesis, embryonic development is affected by its environment. The oviductal time and conditions, the post-oviposition pre-incubation holding environment and time, as well as incubational environment affect the hatchability of the fertile egg (Wilson, 1991). Variability of developmental stage at the moment of oviposition is known, and embryonic development at oviposition may be different for different genetic lines as well for parental age (Decuypere and Michels, 1992). Breed and genetic line differences are known to occur for heat stress susceptibility of immature and adult birds and would be expected to occur also for embryos (Ande and Wilson, 1981). This may also be a direct effect of genetically determined speed of early cell division and development or it may be indirectly linked to variations in oviductal time and/or body temperature (Decuypere and Michels, 1992). It is established that the age of the parental breeder flock has an effect

upon embryonic survival and subsequent chick livability. Investigation with different broiler breeder flocks have shown that the reduced hatchability displayed by eggs from very young breeders was associated with an abnormal distribution of overall embryonic weight; chick weights from eggs of the same weight were lower from young breeders than from older ones. The parent age might influence the use of yolk lipids in the developing embryo, the regulation of heat production because of triiodothyronine metabolism (Weytjens et al., 1999).

There is evidence that, in poultry throughout the whole range of environmental temperatures there are physiological responses that affect their productive performance and efficiency of feed utilization (Al-Saffar and Rose, 2002). McDaniel et al. (1995) mentioned that elevated ambient temperatures during the summer months greatly decrease fertility in broiler breeder flocks. They observed that elevated ambient temperatures decreased hen feed consumption and egg production. Al-Saffar and Rose (2002) observed that breeders exposed to temperatures of about 28-30°C showed a rapid fall in egg production, feed intakes and shell strength. Heywang (1944) showed that the fertility and hatchability of their eggs are decreased when chickens are subjected to high environmental temperatures. That study found a highly significant relationship between intensity of egg production and maximum fertility, and offered the explanation that copulation is decreased when the rate of ovulation is low.

The results may vary by age, genetics, length of stress, and sex. Older males may be more susceptible to stress treatment. Sperm egg penetration decline may be due to sperm motility (McDaniel et al., 1995). Semen quality was decreased during the summer months when Barred Plymouth Rock males were kept at normal, or non-artificial,

summer temperatures (Heywang, 1944). Another factor of sperm-egg penetration may be due to sperm storage tubule release of sperm or lack of binding capacity of the sperm (McDaniel et al., 1995). In females, temperatures affected not only feed consumption and fertility, but also increases in environmental temperature gave relatively small decreases in the egg yolk and eggshell of laying hens until a critical temperature was reduced. There were negative linear regression between eggshell thickness and specific weight of the eggs and increasing environmental temperatures, and a positive linear response with egg deformation (Al-Saffar and Rose, 2002). With reduction in reproductive quality and production in male and females it is difficult for an embryo to survive changes in temperatures. Reproductive traits are factors of breeder management that might affect embryo survival, but another factor that affects embryo survival is the hen's nutrition.

The hen's nutritional status affects the amount of nutrients available during egg formation (Paton et al., 2002a). The inclusion of vitamins and minerals in the hen's diet increases the availability of these nutrients to the embryos. An important nutrient added to breeder diets is selenium (Se). Se source and dietary inclusion level influences the Se concentration of parts of developing embryonated eggs (Cantor and Scott, 1974). The essentiality of selenium for prevention of exudative diathesis and pancreatic fibrosis in growing chicks has been well established (Cantor and Scott, 1974). Whole egg Se is directly influenced by the level of Se in the maternal hen's diet (Paton et al., 2002b.). Latshaw and Biggert (1981) showed that with supplemental Se, there was an increase in both yolk and white proteins in the egg. Paton et al. (2002b) showed that maternal dietary Se had a significant impact on Se content of the total egg, embryo and extra-embryonic portions of eggs incubated for 5, 10, 15 or 20 days. Se content of the hen's

diet has been shown to influence the amount of Se concentration and the activity of GPx in the newly hatched chick (Paton et al., 2002a).

Maternal diet composition is a major determinant of antioxidant system development in the chick during embryogenesis and in early post-natal development. Vitamin E is effectively transferred from food into egg yolk (Surai, 2000). Christensen et al. (2002) showed that adding dietary iodide to turkey hen diets, improved embryonic livability. These are just a few examples on how breeder diets might influence the embryo's resistance to stress. These examples are used because they are more closely related to the study, without taking out of consideration many other important factors. Breeder environmental and nutritional management might have a big influence on embryo stress management, but incubation management also plays a big role in the embryo's survival.

C. Effects of incubator management on embryo stress management

The temperature experienced by the developing embryo is dependent on three factors: 1) the incubator temperature, 2) the ability of heat to pass between the incubator and the embryo, and 3) the metabolic heat production of the embryo itself. Clearly the design of the incubator will have an effect on the transfer of heat between the egg and the incubator air (French, 1997a). French (1986) showed that the multi-stage incubator is unable to provide the optimum conditions for each stage of embryonic development because it contains eggs of 4 or 8 different ages. It was also proposed that single stage incubators had many potential benefits over the multi-stage including better disease control easier egg handling and more flexible setting capacity.

Temperature without any doubt is the most important single environmental which has to be considered as a critical in influencing or determining hatchability (Decuypere and Michels, 1992). As the chicken embryo starts to develop it starts to produce it own metabolic heat, such that from approximately day 14 onwards, embryonic temperature is higher than incubation temperature. Based on these observations it was suggested, "constant embryo temperature is more important than constant incubation temperature (French, 1986). Swann and Brake (1990) showed that an increase in dry-bulb temperature may have inhibited internal heat production, therefore affecting growth and water production briefly; a decrease in dry-bulb temperature may have taken a number of hours to have its effect.

Decuypere and Michels (1992) mentioned that low temperature treatment in turkey embryos during the last quarter of the incubation stimulated postnatal growth as well as changing post-hatching endocrine function suggesting that the functional pituitary-adrenal axis is established. Dropping incubator temperature by up to 2°C in the latter part of incubation improved hatchability (French, 1986). It would also appear that the relationship between sensitivity to high incubation temperature does not show a linear decrease with embryo age as some of these studies also found a greater hatch depression with high temperatures during the last days of development (French, 1997b). Incubators require an air conditioning unit to provide heat or cooling and humidification and a fan to circulate the conditioned air through the eggs before being returned to the conditioning unit (French, 1997a).

Although many embryo stress situations involves failure of temperature control equipment, it should be noted that many accidental occurrences of heat stress to embryos also result from electrical power failures that cause additional disruption of the control, ventilation and egg turning systems. Thus, under applied conditions, heat stress may be further complicated by lack of oxygen, excess carbon dioxide, lack of turning, and improper humidity (Ande and Wilson, 1981). To maintain optimal incubation environment air movement, egg turning and egg humidity has to be considered. The rise in air temperature as it passes over the eggs is inversely proportional to air volume flow and there fore uniform control of egg temperature within the incubator depends on uniform air movement (French, 1997a). In non-isothermal conditions incomplete air mixing gives rise to three-dimensional temperature gradients that have major impact on process quality, energy use, and process efficiency (Van Brecht et al., 2003). The incubation process is a highly time variant bioprocess. The eggs evolve from heat requiring organisms to heat producing organisms due to change in metabolic activity. The incubator control strategy needs to be able to cope with this time variability. Therefore, the total airflow pattern in the incubator will be the result of (1) the heat production of the eggs, (2) the heating or cooling control actions, and (3) the fresh air ventilation rate and the recirculation ventilation rate (Van Brecht et al., 2003). The uniformity of airflow within an incubator will depend on how easy it is for the air to pass between the trays of eggs (French 1997a). The effect of the turning is to reduce the space between the trays significantly from the spacing when the trays are horizontal. As the spacing increases, there is an exponential decline in required air speed (French 1997a). Wilson discussed that the practical way to do egg turning is 24 times per day at a 45° angle with the eggs

small end down. Increasing air speeds results in faster air speeds over the eggs and lower temperatures supporting the predictions that air speed has a major influence on thermal conductivity (French 1997a). Temperature and airflow are not the only factors that affect the embryo during incubation. Relative humidity (RH) and shell conductance also play an important role. Swann and Brake (1990) proved this by showing that by raising the RH there was a lower water loss in the egg decreasing eggshell conductance and increasing hatching time. That study also showed that if conductance is affected by increasing or decreasing shell pores. Colloid treated eggs lost less weight than chlorine treated eggs meaning that with fewer shell pores, egg shell conductance decreased; this creates an absence of regulating water loss, increasing time of hatch (Swann and Brake, 1990). Understanding the general physiological behavior of embryos in dealing with stress and the development of management practices to improve the performance of these are very important in poultry production. In order to understand further these processes, and create more assertive decisions on how to manage the problem, the molecular aspect of dealing with stress has to be understood. The following discussion will focus mainly in heat shock protein 70's interaction with the Se-dependent antioxidant enzyme, glutathione peroxidase.

III. Heat Shock Protein 70

A. Overview of Heat Shock Proteins

The temperature of incubation is one of the major environmental factors that affect embryonic development. Chickens and their cultured cells react to stress condition as other vertebrates by expressing heat shock proteins (Leandro et al., 2004). In response to heat shock or various other kinds of stress, all organisms so far examined, from bacteria, yeast, and other microorganisms to plants, insects, and higher vertebrates, such as fish, chicken, mouse, and man, react by the strong activation of a limited number of specific genes previously either silent or active only at low levels. Consequently, the proteins, encoded by these genes, the so-called heat shock proteins (hsp's), are actively synthesized during stress and accumulate in such a manner as to finally represent major cellular constituents (Southgate et al., 1985). Schlesinger (1986) defined hsp's by two criteria: (a) its synthesis is strongly stimulated by an environmental stress, in particular, that resulting from a change in temperature a few degrees centigrade above the normal physiological one, and (b) its gene contain a conserved sequence of 14 base pairs in the 5' non-coding region. This sequence serves as the promoter for hsp mRNA transcription. The heat shock response was first reported as a dramatic change in gene activity induced by a brief treatment of *Drosophila hydei* larvae. Similar findings in other eukaryotes and prokaryotes suggested that the heat shock response was an evolutionary conserved system for cell survival (Mezquita et al., 1998). Voellmy and Bromley (1982) mentioned that the heat shock response, whereby a new set of proteins, the hsp's, are synthesized at slightly elevated temperatures, is of widespread occurrence and has been studied in simple organisms and cultured cells. They observed that hsp's are synthesized at low

rates at normal temperatures and became the predominant protein products at elevated temperatures. It is known that many hsp's are expressed as an emergency response to diverse stressor agents, and that increased synthesis of these proteins seems to be involved in protection of stressed cells and organisms (Gabriel et al., 2002). Hightower (1993) mentioned that as more biologists became aware of the heat shock response, the list of stressors capable of inducing the response grew quite long and diverse. Besides heat, these included heavy metal ions such as Cu, Cd, and Zn, arsenicals, ethanol, amino acid analogs, puromycin, reoxigenation after hypoxia, and others. Lindquist (1986) mentioned that the induction of hsp's in organisms is rapid, but the maximum induction temperature varies, correlating with the normal range of environmental exposure. In salmon you can see induction at 28°C, in sea urchins at 30°C, in corn plants at 40-45, and Halobacteria at 60 (Lindquist, 1986). It can be transient at "moderate" temperatures and sustained at "high" temperatures (Lindquist, 1986). This means that at transient temperatures, there is an induction of the heat shock response but it turns over rapidly; at "high" temperatures, there is a sustained response and turns over more slowly. Members of the super family of hsp are among the most conserved proteins known in phylogeny with respect to both function and structure. The proteins act as molecular chaperones by binding to other cellular proteins, assisting intracellular transport and folding into the proper secondary structure, thus preventing aggregation of proteins during stress (Yahav et al., 1997). Hsp's are usually encoded in small multigene families whose members are closely related by nucleotide sequence (Hightower, 1993). These proteins are divided into families, based on their structural homologies and molecular weights in polyacrylamide gels, and their functions are related to intracellular protein synthesis and transportation,

folding and assembly of protein complexes (Givisiez et al., 2001) Some of the encoded proteins are produced constitutively as essential proteins (frequently called *heat shock cognate proteins*) in normal cells, and some are stress inducible (Hightower, 1993). This section is going to mention some of the families of hsp's and their known functions.

Hsp 110. Both the 100 and 110-kd proteins are constituents of normal cells and are glucose regulated. Their induction patterns are complex: under various conditions they and the so-called glucose-regulated proteins are induced together, independently, or reciprocally (Lindquist, 1986). With longer heat shocks, hsp110 forms a ring-like structure at the nucleolar periphery. Immunoelectron microscopy indicates that hsp110 associates with the fibrillar component of nucleoli, the site of nucleolar chromatin (rDNA). Treatment of fixed cells with RNAse eliminates staining, suggesting that protein associates with RNA or with a complex of proteins that bind RNA. Since ribosome production is very sensitive to heat shock, it is speculated that hsp110 is induced to protect it (Lindquist and Craig, 1988).

Hsp90. Members of the hsp90 gene family have been cloned and sequenced from several evolutionary diverse organisms, including fruit flies, yeasts, chickens, mammals, trypanosomes, and bacteria. The apparent molecular mass of eukaryotic members of the hsp90 family ranges from 80 to 108kd (Mahmoud, 2000). Sequence analysis of the cloned genes demonstrates that the proteins are very highly conserved. The proteins of even the most distantly related eukaryotes have 50% amino acid identity, and all have greater than 40% identity with the *E.* coli protein (Lindquist and Craig, 1988). This protein is synthesized under normal cell culture conditions, and after stress its formation continues at a relatively elevated level; it is localized to the cytosol compartment of the

cell (Schlesinger, 1985); with the exception that it is also found in the endoplasmic reticulum in higher eukaryotes (Mahmoud, 2000). In most cells it is abundant at normal temperatures and induced by heat, but abundance and inducibility vary in a tissue specific way. In yeast, separate genes produce constitutive and inducible forms (Lindquist, 1986). In *D. melanogaster* hsc83, is constitutively expressed at a high level and is moderately heat-inducible; the other, hsp83, is constitutively synthesized at a lower level and is more strongly heat inducible (Lindquist and Craig, 1988). To date, the most interesting property reported for this protein is its transient association with retroviral transforming proteins and steroid hormone receptor complexes. Monoclonal antibodies prepared against the 8S progesterone receptor revealed that hsp90 is a major component of the complex by controlling the strength of the binding of the receptor to the hormone (Lindquist, 1986).

Hsp70. Hsp70 genes were first visualized as puffs on polytene chromosomes isolated from salivary glands of *Drosophila* larvae subjected to high temperature. Proteins related to *Drosophila* hsp70 have been identified in all examined organisms (Mahmoud, 2000). Using monoclonal antibodies, the presence of two abundant proteins encoded by these genes, called hsc70 and hsc72, have been identified (Lindquist and Craig, 1988). There are four genes expressed during normal growth that have been identified as the heat shock cognate genes (Mahmoud, 2000). This is the most highly conserved of the hsp's and, as such, has aroused the greatest interest. The human protein is 73% identical to the *Drosophila* protein and 50% identical to the *E. coli* dnaK product (Lindquist, 1986). Studies have shown that the *E. coli* hsp70 is the product of the dnaK gene and interacts with lambda phage O and P proteins during phage replication

(Schlesinger, 1993). Lindquist and Craig (1998) also mentioned that genetic analyses have shown that dnaK is essential at high temperatures, and perhaps at lower temperatures as well. *S. Cerevisiae* has four genes isolated from a yeast genomic library, which show similarity to the *Drosophila* genes. The transcriptional activity of these four genes show that two of the yeast hsp70 were induced by heat, and the other two were found to be expressed only at normal temperature and to be repressed under heat shock conditions (Southgate et al., 1985). Mammalian hsp's are divided into four groups: hsp70, hsp72, p72, and grp78. Hsp70 is the major inducible protein, hsp72 is a protein expressed only after heat shock, p72 is expressed at high levels in growing cells, and grp78 is a glucose-regulated protein located in the endoplasmic reticulum (Lindquist and Craig, 1985). Mahmoud (2000) mentioned that hsp70 is a cell cycle regulated protein, hsp72 is also known as hsp70 and it is induced after heat shock, p72, which is known as hsc70/hsc72 is expressed at high levels in growing cells and is slightly heat inducible.

All hsp70 and related proteins bind ATP with high affinity; many are very abundant in cells and often found in association with other proteins (Lindquist and Craig, 1988). The function of hsp70 can be described as peptide folding proteins (Mahmoud, 2000). Hsp 70 functions will be describe later in the review.

Hsp60. This protein is also known as stress protein 60 and it serves as a molecular chaperone. A molecular chaperone is defined as a protein that has the ability to assist the non-covalent assembly of other protein-containing structures in vivo, but which are not permanent components of these structures when they are performing their normal biological functions (Ellis, 1996). These have been largely studied in *E. coli* with the GroEL and GroES proteins. These are constitutively expressed in non-stressed cells, and

its level increases two to three folds after heat shock and is mithocondrial associated (Lindquist and Craig, 1988). Langer and Neupert (1996) showed that assist in the assembly of oligomeric protein complexes such as the F1-ATPase complex and the Rieske Fe/S protein. It has also been observed that it assist in the folding of polypeptides such as dihydrofolate reductase and it assists hsp70 in the translocation of newly synthesized proteins through the mitochondria membranes.

Small hsp's. The small hsp's family is a very diverse group ranging in size from 15 to 40kd (Mahmoud, 2000). Different organisms have different numbers of small hsp's, ranging from one, in *S. cerevisiae*, to upwards of 30 in higher plants (Lindquist and Craig, 1988). A role for the small hsp's has not been found. Schlesinger et al. (1989) found that small hsp's can aggregate with itself to form huge polymeric structures. It has been suggested that small hsp's may behave as a microfilament protector (King et al., 2002). Lindquist and Craig (1988) mentioned that there is evidence that they may serve to preserve inactive mRNA. Mahmoud (2000) mentioned that there are several studies that induction of small hsp's are related in the acquisition of thermotolerance in *D. melanogaster* and tomatoes. Several studies have agreed that small hsp are regulated in a tissue and stage specific manner (Lindquist, 1986).

Ubiquitin. Ubiquitin, a highly conserved 76-residue protein, which as its name implies is found in all eukaryotic cells, is induced by heat (Lindquist and Craig, 1988). It is an essential component of the stress response system, specifically required for cell survival under a broad range of physiological stresses (Mezquita et al., 1998). In both chickens and yeast, ubiquitin has been found to be heat inducible (Lindquist, 1986). At the non-permissive temperature synthesis of ubiquitin and some members of the hsp70

family is increased (Lindquist and Craig, 1988). Heat stress leads to a sudden increase in the level of damaged proteins that can be toxic to the cell. Covalent conjugation of ubiquitin to damaged proteins can trigger their degradation (Mezquita et al., 1998). Schlesinger et al. (1989) showed that there was little change in protein turnover during stress, but turnover rate double immediately upon removing cells from stress. Subsequently they mentioned that the major mechanism for removing denatured proteins involves the ubiquitin-dependent proteolysis pathway in which proteins targeted for degradation are conjugated with multiple ubiquitins.

Hightower (1993) mentioned that at least three size classes of hsp's, hsp90, hsp70, and hsp60, all have chaperoning functions. They are normally involved in protein folding/unfolding pathways, and in intracellular movement of proteins within the cytoplasm and across membranes. Yahav et al. (1997) mentioned that the hsp110, hsp90, hsp60, and the small hsp's contain both proteins that are present before heat treatment, and proteins whose synthesis becomes detectable only following stress. All of these proteins are very important before, during, and after stress, but hsp70 is the most abundant and more related to heat stress tolerance. That is why the main focus of this study was the influence of this protein on turkey embryo development.

B. Heat shock protein 70

a. General Characteristics.

The cells, in culture or in vivo, respond to stress by decreasing significantly the synthesis of almost all cellular proteins except a selected group of highly conserved proteins, the heat shock protein 70 (Leandro et al., 2004). Exposure of poultry species to mild stressors over a period of time enhances hsp70 expression, but eventually, the birds become acclimated and no further increase in cellular hsp70 can be demonstrated (Edens et al., 2001). All hsp70 and related proteins bind ATP with high affinity; many are very abundant in cells and often found in association with other proteins (Lindquist and Craig, 1988). This multifunctional molecule, when under inducible regulatory expression, exhibits a broad range of chaperone functions that respond to both internal and external stresses (Norry and Loeschcke, 2003). It has been suggested that hsp70 might be involved in cellular protection in adverse situations, and a relationship between the development between this protein and thermotolerance has been established (Givisiez et al., 2001). These are induced by a variety of stresses (hyperthermia, ethanol, amino acid analogs, heavy metals, free radicals, etc.), and are believed to play a critical role in protecting cells from these stresses (Van Remmen et al., 1996). Lindquist and Craig (1998) mentioned that with the use of monoclonal antibodies, two forms of the hsp70 were identified: a constitutive form (hsc70) and a heat inducible form (hsp70). Hsc70 in unstressed cells is distributed in the cytoplasm and nucleus. Hsc70 sequences are more highly conserved among mammals (Hightower and Leung, 1997). These proteins along with newly synthesized hsp70 increase in the nucleus and then redistribute to the cytoplasm during recovery. (Pelham, 1990; Hightower and Leung, 1997). Both of these

proteins are concentrated in regions active in protein synthesis in cells and may bind to nascent polypeptide chains (Hightower and Leung, 1997).

b. Induction of hsp70

As discussed above, hsp70 is induced by a variety of agents or stressors. Heat is the most prominent inducer. Heat pre-conditioning is related to the expression of hsp's and antioxidant enzymes (Hoshida et al., 1997). Wang and Edens (1994) found that continuous daily exposures to sub lethal heat stress for 1, 3 of 5 weeks was sufficient to allow those birds to induce more hsp70 mRNA as well as hsp70 protein under an acute heat environment. Wang and Edens (1997) also found that hyperthermia of 45.4°C led to a significant induction of hsp70 in broiler chickens.

There are several different ways to induce hsp70 in organisms. Whelan and Hightower (1985) studied the effects of pH and sulhydryl redox state, and proposed that inducers of oxidative stress induce hsp70. They observed that basic environment is sufficient inducer of hsp70 because of a high hydroxyl radical that disrupts sulfhydryls groups of proteins. Abe et al. (1994) showed that Cadmium (Cd) has high affinity for sulfur; some toxic effects of Cd are results of reactions with essential sulfhydryl groups in proteins. Abnormal proteins cause the activation of hsp70 gene. Ethanol induces hsp70 in mammals and *E. coli*. Sodium arsenite induces the proteins in *Drosophila*, mammals, trout, and soybeans. Anaerobiosis and recovery from it also induces the proteins in many organisms (Lindquist, 1986). Other inducers include amino-acid analogs, sulfhydryls reactive chemicals, and certain metal ions such as Fe, Cd, and Cu (Whelan and Hightower, 1985).

The induction of hsp's in response to cellular stress is mediated by a family of heat shock factors (hsf), that act through a highly conserved promoter element upstream of hsp genes referred to as heat shock element (hse) (Mahmoud, 2000). Upon heat shock, the chromatin structure changes profoundly. First, the coding region becomes more accessible to nucleolytic attack. Second, the position and number of hypersensitive sites changes. Most notably, regions centered on the hse become refractory to digestion, denoting the binding of protein (Lindquist, 1986). At least two hsf's are present in mammals, which are present as monomers and during stress it changes conformation and form trimers (Hightower and Leung, 1997). The induction of hsp70 expression in response to hyperthermia is mediated by the binding of the hsf to a highly conserved DNA sequence known as the hse. In non-stressed cells, hsf exists as a monomer and oligomerize upon heat shock to bind to the hse (Van Remmen et al., 1996). Manipulation of the transcription and translation process can affect cell tolerance to heat. Johnston and Kucey (1998) showed that by mutating hamster ovarian cell inhibited binding of the hsf, inhibiting transcription, and by producing antibodies against hsp70, inhibited the production of hsp70 protein.

Hsp70 expression is tissue and age specific. Aréchiga and Hansen (1988) showed that preimplantation murine embryos, do not exhibit heat shock tolerance because of lack of expression of hsp70. Edwards et al. (2001) mentioned that exposure of embryos to increased culture temperature decreases development. Acquisition of thermal tolerance may be contingent upon acquisition of protective biochemical mechanisms or simply due to increased cell numbers (Edwards et al., 2001). One reason for the heighten sensitivity of the early embryo to stress may be the fact that the genome is largely suppressed dung

early cleavage stages (Al-Katanani and Hansen, 2002). Mirkes et al. (1999) showed that that day 8 mouse embryos express hsp70, and they are protected from the embryo lethal effects of hyperthermia, as evidenced by the observed increase in viability among embryos. Givisiez et al. (2001) observed that chicken embryos expressed different levels of hsp70 expression in different tissues. Leandro et al. (2004) showed different tissues were susceptible to heat stress at different age, and that hsp70 expression dependent on tissue susceptibility at different ages. Heydari et al. (1996) showed that adult mice decreased hsp70 expression with increasing age compared to young mice. Induction is also affected by selection for heat tolerance as studied by Norry and Loeschcke (2003), who found that selection for longevity and heat tolerance in *Drosophila* increased the temperature threshold for hsp70 induction.

The rule for heat shock genes appears to be that stressors must cause the accumulation of damaged proteins in cells. This damage is monitored within cells and results in activation of preexisting regulatory proteins called hsf's (Hightower, 1993). After induction hsp70 has been associated with induction of thermotolerance using chaperoning functions.

c. Hsp70 functions

Hsp70 functions as a molecular chaperone. Molecular Chaperones are proteins that facilitate the folding or assembly and disassembly of other proteins but are not part of the finished structure (Hightower et al., 1994). One possible mechanism may involve on of the well-known chaperone functions of heat shock proteins, that is the ability to protect proteins from denaturation (Mirkes et al., 1999). Hsp70 also acts as a molecular

chaperone by participating in folding, transport and post-translational modification of proteins (Al-Katanani and Hansen, 2002). Lindquist and Craig (1988) discussed that hsp's might bind to denatured or abnormal proteins after a heat shock to prevent their aggregation and thus prevent cellular damage. Hsp70 and related proteins have a high affinity of ATP (Lindquist and Craig, 1988). Mahmoud (2000) discussed that The N-terminal of the protein has ATPase activity, while the C-terminal bind proteins through their peptide binding domains. Pelham (1990) described the mechanism of action for the hsp70 proteins. The substrate of the protein would be newly synthesized or recently denatured proteins that are capable of folding to their tertiary and quaternary structures. These structures have hydrophobic regions that might aggregate with other proteins to form non-functional precipitates. Hsp70 binds to these hydrophobic regions to reduce such regions and inhibit the aggregation process. Hsp70 hydrolyzes an ATP molecule and undergoes a conformational change that decreases its affinity for the substrate. If there are still hydrophobic regions, the cycle is repeated, if not hsp70 interact with each other to form dimers. Hightower et al. (1994) discussed that the current chaperoning mechanisms for the hsp70 family include (1) binding to nascent polypeptide to delay folding until all of the information needed for folding is present, (2) delaying folding until reaching its appropriate cellular compartment, (3) blocking aggregation, and (4) membrane translocation. All of these mechanisms are induced to protect the cell from environmental insults and induce thermotolerance.

Thermotolerance refers to the state of resistance to hyperthermic conditions that can be improved by acclimation (Moraes et al., 2003). Organisms exposed to temperature elevation induce hsp's. In a remarkable range of cells and organisms, incubation at

temperatures that induce the hsp's produces tolerance to much more extreme temperatures. Sub-lethal heats treatments induce developmental anomalies in many organisms, and preheat treatment reduce or eliminate these defects (Lindquist and Craig, 1988). Han and Li (1990) proposed that the thermotolerance mechanism is induced upon heat shock and converts normal cells to triggered cells, which during prolonged treatment, the cell is converted to a thermotolerant cell. Failure to keep challenging the cell causes a degradation of the response and the cell turns back to a sensitive stage. Lindquist (1986) mentioned that the kinetics of thermotolerance induction, under many different conditions, are tightly correlated with the kinetics of heat shock protein synthesis and reaches a maximum when the accumulation of hsp's plateaus. Also the decay of thermotolerance coincides with the degradation of hsp's. Wang and Edens (1998) showed that daily exposure to heat conditioning environment for 1 week was sufficient to enhance the heat shock response in chickens and about 3 weeks for turkeys. Yahav et al. (1997) showed that synthesis of hsp70 mRNA is induced to a lesser extent in tissues of broiler chickens, which acquired improved thermotolerance, than in tissues of control chickens.

Non -thermal stressors can induce tolerance. These include arsenic, Cd, ethanol, hypoxia, and glucocorticoids (Wang and Edens, 1998). Exposures to heavy metal ions also induce tolerance by induction of hsp's. Heat exposure reciprocally induces resistance to these stressors (Lindquist and Craig, 1988). King et al. (2002) hypothesized that hyperthermia induced various physiological responses, which in turn triggered hsp70 synthesis. Some of these responses include the release of stress hormones, cytokines and electrolytes, and reduced oxygen partial pressure. For example, Wang and Edens (1994)

found that increased levels of plasma testosterone and corticosterone induced hsp70 in large-comb birds, which were more tolerant. King et al. (2002) showed that reactive oxygen species that is caused by metabolic perturbations also induce hsp70.

Hsp70 is the protein mostly associated with thermotolerance, and it has been supported using competitive inhibition of hsp70 expression, which increased thermosensitivity (Wang and Edens, 1994). There are other factors that may contribute to thermal resistance in organisms. Antioxidants for example have shown to reduce ROS, which are inducers of hsp70. King et al. (2002) showed that, after heat exposure, Superoxide dismutase (SOD) activity is increased. Other hsp's are also induced such as ubiquitin and small hsp's, which help in the elimination of denatured proteins and the reestablishment of normal cellular process (Schlesinger, 1986). Longevity selection has also shown has also an effect. Norry and Loeschke (2003) showed that short-lived individuals are very susceptible to heat stress in terms of hsp70 induction. Development also has an effect as Al-Katanani and Hansen (2002) showed, that bovine blastoderm showed can undergo induced thermotolerance, and the 2-cell embryo cannot because of its inability to produce hsp70.

Hsp70 induction would lead to protection from stress in embryos. As discussed above, heat stress stops the majority of protein production except hsp70. Shallom et al. (2002) showed that enhanced hsp70 in chick embryos lead to increased cytoprotective effects from hypoxic stress. Gabriel et al. (2003) observed that increases in temperatures increased hsp70 mRNA levels causing little effect on myogenesis, so hsp70 might have a protective effect on synthesized proteins during development. Mirkes et al. (1999) also reported that heat-induced tolerance to oxidative injury is correlated with an hsp70-

mediated protective effect on mitochondria. Thus, hsp's may exert their protective effects through multiple pathways that impinge on the apoptotic cascade.

IV. Selenium

A. Overview of Selenium

Selenium is an essential trace element discovered by J.J. Berzelius (1817), in Stockholm, Sweden. The biological significance was not recognized until 1856, when it was identified as the toxic agent associated with alkali disease, now called selenosis, and in 1957, it was accepted as an essential trace nutrient (Edens, 2002). Se exists in two chemical forms, organic and inorganic. Inorganic Se can be found in different minerals in the form of selenite, selenate and selenide as well as in the metallic form. Organic Se can be found in forages, grains, and oilseed meals, bonded to different amino acids including methionine and cysteine (Surai, 2002).

It has been recognized that the selenoamino acids, selenomethionine, selenocysteine and selenocystine, are the primary sources of selenium in plant-based and meat-based ingredients (Edens, 2001). Latshaw and Biggert (1981) mentioned that the transport across the intestinal wall is similar to that of methionine. The organic selenium is readily available and is actively absorbed from the intestine via the Na-dependent neutral amino acid pathway (Edens, 2001). Selenomethionine has shown to be incorporated into protein, presumably by peptide bonds and by substituting methionine (Latshaw and Osman, 1974). In cases where selenomethionine is at a high level in feeds, it can be demonstrated that 40 to 50% of total body Se as selenomethionine can be found in muscles (Edens, 2002). Selenomethionine is easily converted to selenocysteine via

cysthionase. Selenocysteine is the main selenoamino acid found in selenoproteins, such as glutathione peroxidase. Selenocysteine can substitute for cysteine in proteins (Edens, 2002). In order for selenocysteine to be incorporated into specific selenoproteins, selenocysteine-β-lyase must react with free selenocysteine to release selenide in the presence of reducing agents. Then, selenocysteyl- tRNA [Ser] ^{SEC}, which recognizes the specific UGA stop codons in the selenoprotein-mRNA, inserts the new, contranslationally-synthesized selenocysteine into the specific selenoprotein (Edens, 2002).

Selenocysteine can be found in the body of animals fed inorganic Se such as selenite and selenate. Selenite absorption is by diffusion (Latshaw and Biggert, 1981). The synthesis of selenocysteine involves a unique process in which selenide is phosphorylated under the influence of selenophosphate synthetase to selenophosphate. This is made available to a seryl-tRNA^{SEC} that is recognized by selenocysteine synthetase, and converts it to selenocysteyl-tRNA^{SEC}, which allows insertion of selenocysteine into the peptide chain as described above (Edens, 2001). Selenite has been shown to be a prooxidant by interacting with glutathione generating a superoxide radical Selenite is potentially toxic due to reactions with glutathione and possibly other sulphydryl compounds present in biological systems that ultimately produce ROS. Selenomethionine does not participate in prooxidant reaction and possesses antioxidant properties (Surai, 2002).

It has been shown that the selenium source has an effect on its concentration in eggs. Combs and Scott (1979) showed that supplemental selenium significantly increased the deposition of selenium in egg contents. Latshaw and Osman (1974) showed that there

has a higher accumulation of selenium in the egg white than in the yolk when they were fed organic selenium and vice-versa when they were fed sodium selenite. Latshaw and Biggert (1981) had similar results and concluded that this is because egg white proteins had higher cysteine content. Paton et al. (2002b) showed that eggs from hens fed organic selenium had a higher selenium content in the eggs than hens fed inorganic sources, and that subsequently embryos from organic selenium fed hens had higher Se concentration in tissues than embryos from selenite fed hens. Paton et al. (2002a) contradicted the results from Latshaw and Biggert (1981), and Latshaw and Osman (1974) by comparing different studies in which they showed that yolks with organic selenium had a higher content than yolks with sodium selenite. They also showed that selenium from selenite in eggs plateaus with dietary selenium between 0.3 and 1.0 ppm, but organic Se in eggs continually increased with increasing levels of supplemental Se. Edens (2001) discussed that organic Se is superior to sodium selenite in terms of induction of feathering, in tissue accumulation and retention in broilers, and reducing drip loss from breast meat.

Arnold et al. (1972) showed that within 12 days, the selenium content of the eggs had reached a maximum of about 1.7 ppm. Which means that dietary inclusion of Se in hen's diets will increase the deposition of Se in the egg. This has positive effects on embryo development. Paton et al. (2002a) discussed that embryos from hens fed diets containing supplemental Se, contained greater concentrations of Se than embryos from negative controls. Se is deposited mainly in egg yolks (Magat and Sell, 1979). The egg yolk is the major source of minerals (except Ca) for the developing chick and as such readily accumulates the necessary minerals for inclusion into the egg (Paton et al., 2002a). There are significant linear increases in embryo Se concentration during

incubation with the most obvious increase between days 10 and 15. Surai (2000) showed that there is tissue specificity in selenium transfer from the egg to the embryo. For example, in contrast to the liver, there was only a trend for Se accumulation in the brain of chickens. Paton et al. (2000) observed that Se source and dietary inclusion level influences the Se concentration of parts of developing embryonated eggs. Surai (1999) showed that the yolk Sac membrane had maximum antioxidant enzyme activity on day 15 of development. It is probable that increased activities of GPx and catalase at day 15 and a sharp increase in peroxide accumulation at day 16 of development indicate oxidative stress at this stage. Surai (2000) mentioned that accumulation of natural antioxidants in the liver during embryogenesis is considered to be an adaptive mechanism to protect against lipid peroxidation during hatching time and early post-natal life. Surai (1999, 2000) also showed the sparing effect of selenium on vitamin E metabolism and transfer into the egg yolk and the developing tissues. For example, an inclusion of organic selenium in the breeder diet significantly increased vitamin E concentration in egg yolk. Diplock (1976) found that selenium spares vitamin E in three ways. First, it preserves the integrity of the pancreas, which allows, indirectly, proper absorption of vitamin E. Second, selenium aids in the retention of vitamin E in blood plasma. Third, it may decrease the chance of oxidative damage from peroxides via its function in glutathione peroxidase. Another finding from that same study was that selenium supplementation might help maintain high levels of reduced glutathione (a water soluble antioxidant). Cantor and Scott (1974) showed that selenium supplementation to breeder hens increased hatching and survival of offspring. They also showed that newly hatched

chicks from selenium-supplemented hens had a higher survival rate when fed a selenium deficient diet compared to control birds.

In general Se supplementation has many positive effects in broiler production. Rutz et al. (2003) showed that in layers, selenium supplementation improves egg production, egg weight, internal quality of eggs, shell quality, yolk and albumen weight, and feed conversion. In broilers, there is an improvement in body weight gain and fed efficiency; and in breeders, there is an improvement in hatchability and chick quality. Edens (2001). Also mentioned that there is an improvement in feathering, higher triiodothyronine levels. Edens (2002) also mentioned that there is an increase in sperm quality.

Selenium (Se) also has its detrimental effects by its toxicity and deficiencies. Se deficiency in the chicken, especially in combination with low vitamin E supply, is responsible for the development of a range of diseases including exudative diathesis, nutritional encephalomalacia, and nutritional pancreatic atrophy, which is the only clearly defined Se deficiency uncomplicated by deficiencies of other antioxidants (Surai, 2002). Se has been reported to delay the effects of encephalomalacia and muscular dystrophy in chickens. Se responsive diseases in the turkey poult include smooth muscle myopathy, myocardial myopathy (Diplock, 1976). Severe Se deficiency leads to affect the thyroid hormone by inhibiting iodide oxidation into iodine, causing an increase in corticosterone levels. Corticosterone levels inhibit bursa of Fabricius growth (Jensen et al., 1986). In livestock and experimental rodents, Se deficiency leads to alterations of spermatogenesis, impairment of sperm mobility, morphological alterations of the midpiece architecture resulting in disconnections of heads and tails (Foresta et al., 2002). Intoxication with

other metals like lead (Pb) eliminated the growth response to added Se in chicks. The antagonism of Pb makes a portion of the dietary Se unavailable (McGowan and Donaldson, 1987). Se deficiency is associated with impaired immunocompetence, reduced egg production, increased embryonic mortality, and reduced hatchability (Surai, 2002).

Se is toxic when used in high doses (about 10 times the recommended amount), but this varies by species, age, and Se source (Surai, 2002; Fitzsimmons and Phalaraksh, 1978; Palmer et al., 1973). Se toxicity in animals can cause mainly fatty degeneration of the liver. Other complications include liver cirrhosis, abnormal posture, bloating, and cyanosis (Diplock, 1976). Selenite toxicity induced lipid peroxidation in rat heart (Padmaja, 1993). Palmer et al. (1973) observed that when chicken embryos were injected with toxic levels of different selenium sources several abnormalities were observed. These included, underdevelopment of the beak, feet, and legs, including webbing of the two outside toes. Growth depression and reduced egg production, anemia and stiffness of the tibiotarsal joints, increased liver and heart weight are related to Se overdose.

The main function of Se is in the function of the selenoenzyme glutathione peroxidase (GPx). The importance of this enzyme in birds will be discussed in the following section.

B. The Role of Selenium on Oxidative Stress

Membranes of sub-cellular organelles contain relatively high concentrations of polyunsaturated lipids as well as hemoproteins, which are strong catalysts of lipid peroxidation (Padmaja, et al., 1993). Surai (1999) showed that different tissues of the embryo display distinct development strategies with regard to the acquisition of antioxidant capacity. Chicken embryos are characterized by high concentrations of polyunsaturated fatty acids and are very sensitive to lipid peroxidation (Surai et al., 1997). A low oxygen pressure in embryos seems to be a protective mechanism of lipid peroxidation of vulnerable tissues. It is suggested that an accumulation of natural antioxidants, vitamins A and E and carotenoids, as well as an increase in GPx, superoxide dismutase (SOD), and vitamin C activity are developed to protect tissues from peroxidation (Surai, 1999).

Flohé (1999) mentioned that there are four types of glutathione peroxidases: cytosolic GPx (cGPx), plasma GPx p (GPx), gastrointestinal GPx (GIGPx), and phospholipid hydroperoxides GPx, (PHGPx). The cGPx is the most abundant GPx studied and all cells express this enzymes activity. This enzyme inhibits cell apoptosis induced by hydroperoxides. The pGPx enzyme is mainly found in plasma, but it has also been found in mammalian placenta, and kidneys. It functions as a redox buffer by silencing lipooxigenases, and prevents the amplification of phagocyte activity. GIGPx is found mainly in the GI tract and the liver this is the first line of defense against ingestion of lipid hydroperoxides by reducing the amount transported from the gut lumen to the lymph. PHGPx is mainly located in the testis suggesting a function in male fertility. This enzyme has antioxidant function as well as plays a role in redox regulation, sexual

maturation, and differentiation. It reduces hydroperoxides in HDL and LDL. Its inactivity in mature sperm indicates that it serves as a structural protein of the mitochondrial capsule of the spermatozoa.

In general GPx is located in the cytosol and has a general specificity in detoxification of both lipid hydroperoxides as well as organic hydroperoxides (Padmaja et al., 1997). The antioxidant system of the chick embryo consists of a combination of natural metabolites, and the antioxidant enzymes, GPx, SOD, and catalase (CAT). GPx had the highest activity at all stages of development especially in the liver (Surai et al., 1997). CAT peaked at day 10 of embryonic development and after hatching. SOD activity was mainly located in the brain (Surai, 1999). Increases in the activity of GPx in the developing chick would require additional Se in the diet of the hen, and it has been demonstrated that GPx activity in the liver rises quickly after days 10 and 15 of incubation. This is correlated with the development with glial cell in the brain after day 10, which requires GPx protection (Paton et al., 2001a).

Lipid peroxides destroy membrane integrity and decrease membrane fluidity and elasticity (Padmaja et al., 1993). Lipid peroxidation is a complex process in which oxidation of polyunsaturated fatty acids of membrane lipids leads to membrane damage and cell death (Padmaja et al., 1997). Also high temperature can adversely affect the structure and physiology of the cells, causing impairment of transcription, RNA processing, translation, oxidative metabolism, membrane structure and function. Radical generation is a natural consequence of living in an oxidizing environment (Altan et al., 2003). Cells generate small amounts of free radicals or reactive oxygen species (ROS) while performing their normal metabolic functions. Although low levels of ROS are

essential in many biochemical processes, accumulation of ROS may damage biological macromolecules (Altan et al., 2003). External factors such as heat, trauma, radiation, hyperoxia, toxins, and exercise can lead to increased free radicals and other ROS (Altan et al., 2003).

GPx catalizes the preferential oxidation of glutathione by peroxides. Glutathione is a small molecule, a tripeptide (glutamate, cysteine, and glycine), commonly distributed in all eukaryotic cells (Mahmoud and Edens, 2003). NADPH then reduces the oxidized glutathione in a reaction catalyzed by glutathione reductase (GR). Many cellular oxidations have been shown to generate active superoxide radicals ($O_2^{\cdot}$) by the univalent reduction of oxygen. SOD reduces the superoxide radicals to less toxic H_2O_2, which in turn is catabolized to water by CAT and peroxidases (Padmaja et al., 1997). Surai (2000) showed that Se is an important determinant of antioxidant system efficiency because of the increases in GPx activity, which removes lipid and hydrogen peroxides formed during metabolism and superoxide radical dismutation. Altan et al. (2003) also showed that exposing birds to heat stress increased SOD, CAT, and GPx activities. Synthesis of glutathione (GSH) is required during development. Depletion of this molecule increased death and malformation in rat embryos. Tolerance to various cells types to elevated temperature has been correlated with intracellular GSH levels (Edwards et al., 2001). Van Remmen et al. (1996) observed that the GSH/GSSH ratio decreased in the cultured hepatocytes and the level of cultured hepatocytes increased. Higher glutathione peroxidase activity will catalyze oxidation of GSH to GSSG. Reduction of GSSG to GSH is catalyzed by GR which could be rate limiting if there is high amounts of GSSG (Edens et al., 2001).

Heat stressed, Se supplemented broilers showed greater BW, improved FCR, and reduced mortality compared to birds not supplemented with selenium. Also animals subjected to an *E. coli* challenged or heat stress, Se-supplemented birds showed lower induction of hsp70 than non-supplemented birds and higher GPx activity (Edens et al., 2001). Enriching diets with highly oxidated fat and a higher content of Se (0.3 mg/kg), vitamin E (30-40 mg/kg) and vitamin A (1200-15000 IU/kg) does not worsen turkey's health although it lowers vitamin E reserves. Oxidized fat causes reduction of body weight gain of turkeys without symptoms of muscular dystrophy; it also increases susceptibility to infections of virus causing hemorrhagic intestinal inflammation (Zdunczyk et al., 2002). Ando (1994) observed that as GPx peroxidase activities were greatly induced in rat liver, lipid peroxidation in intracellular structures, such as mitochondria and microsomes, were not affected in hyperthermia. On the other hand, that same study showed that hepatic GPx activities were not induced in guinea pig, therefore the marked lipid peroxidation was observed in mitochondria and microsomes in hyperthermia. Selenium has been shown to reduce these effects as described above through an increase in GPx activity. Therefore GPx activity can be used as an indicator of Se content in the cell.

The discovery that GPx contained an integral and stoichiometric quantity of Se demonstrated a biochemical role for this essential trace element and provided a tool for monitoring its status in all animals (Edens, 2002). Glutathione peroxidase is a molecule with 4 subunits of 22kd each. Each subunit contains 1g atom of Se, so GPx contains 4g atoms of Se in each molecule (Diplock, 1976). McGowan and Donaldson (1987) showed that GPx activity was a good indicator of Se status by showing that Se addition helped

overcome GPx inhibition by mercury supplementation. Surai (2000) showed that GPx livers of newly hatched chicks depended on Se supplementation and Se concentration in the tissue. Dietary Se supplementation (0.2 and 0.4 mg/kg) increased GPx activity in the liver of the day-old chicks. The embryo Se concentration begins to increase at day 12.25 irrespective of source or level of Se inclusion in the maternal diet. This implies that, the developing chick has an elevated requirement for Se beginning on or about day 12 of incubation. Especially because the adenophyseal-thyroid axis and the brain's glial cells which need GPx to be fully functional (Paton et al., 2002a). Combs and Scott (1979) showed that after 20 weeks of feeding, pullets fed the low-selenium basal diet showed a deficiency of the Se-dependent glutathione peroxidase, which responded to Se supplementation of that diet. Cantor and Scott (1974) also showed that Se deficiency caused a marked drop of GPx in liver and in plasma in 6-day-old chicks.

Selenium supplementation increases GPx activity in chicken embryos, which reduces physiological stress in embryos, which in turn, affects hsp70 expression. The purpose of this study is to observe how would Se supplementation of turkey breeder hens will affect hsp70 expression in heat stressed turkey embryos. The main objectives of this study were to observe if hsp70 expression in heat stressed turkey embryos change after Se supplementation of the maternal diet. Another objective was to establish a relationship between GPx activity and hsp70 expression. This would help on the understanding on how would embryos manage temperature changes at a molecular level.

Materials and Methods

I. Experiment 1

A. Animal Husbandry

Fifty-two Nicholas turkey breeder hens were assigned randomly between two treatments in a total of 10 pens. Each pen held 5 to 6 hens, and at the beginning of the lighting program, they were fed one of two dietary treatments. One dietary treatment had no supplemental selenium (Se); the other had a supplement of 0.3 ppm of organic Se in yeast[1]. The Se contents of the non-Se-supplemented diet were 0.662 ppm, and the Se content for the Se-supplemented diet was 0.965 ppm. The hens were fed these diets for three weeks to assure that there would be differences in body Se concentrations before they were artificially inseminated (A.I.).

The A. I. program started 17 days after photo stimulation. The hens were given A. I. two times in the first 5 d. After the second A. I., the hens were subjected to A. I. once a week for an additional four weeks. The first eggs were collected one week after the second A. I. Each trial was set at the end of every week after A. I. They were stored no longer than one week.

B. Incubation and stress treatments

A total of 80 eggs per trial were set weekly for five weeks. There were 40 eggs from the non-supplemented treatment, and 40 eggs from the organic Se-supplemented treatment were incubated at 37.5°C for 21 days. The eggs were candled at incubation d18 to ascertain if a minimum of 30 eggs per treatment remained. At incubation d22, the eggs

[1] Sel-Plex®, Alltech Inc., Nicholasville, KY 40356

were subjected to an acute heating episode at 40°C for 2h. The method imitated a common temperature deviation found in commercial hatcheries described by French (1997).

C. Protein determination and electrophoresis for hsp70

Livers from two embryos were separated into left and right lobes and were pooled to make approximately 5g samples. One lobe sample was used for hsp70 analysis and the other lobe sample was used for glutathione peroxidase activity analysis. Liver samples were collected in 4mL cryovials[2] and frozen in a methanol-dry ice bath. The samples then were stored at-70°C until assayed.

Samples were minced and washed three times in 50mL polypropylene tubes using 5mL of lysis buffer (20mM Tris-HCl, pH 7.5; 0.9% NaCl; 2mM β-mercaptoethanol) (Givisiez, et al., 2001). They were then homogenized on ice with an ultra-turax homogenizer[2] at 20,000 rpm. The samples were kept at 4°C during preparation. The samples were centrifuged in a Beckman model J-6B centrifuge[3] at 5,000 rpm for 30 min in 4°C. After the first centrifugation, 1mL of the sample supernatant was transferred to a 1.5mL microcentrifuge tube. The samples were then centrifuged in an IEC micromax RF microcentrifuge[4] at 15,000 rpm for 30 min at 4°C. After the second centrifugation, 500 μL of sample was collected and transferred to a 1.5mL microcentrifuge tube for total protein analysis and for hsp70 determination.

[2] Thomas Scientific, Sweedesboro, N.J. 08085
[3] Beckman Instruments, Inc., Palo Alto, Ca 94304
[4] Thermo Electron Corporation, Woburn, Ma 01801-1086

Total protein analysis was done by the method described by Bradford (1976). A standard curve was prepared using bovine serum albumen samples in concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5 mg/mL. The protein samples were diluted in PBS to a 1:20 dilution rate. Standards and samples (10 μL) were loaded in triplicate into microplate wells. Then, 200μL of a 1:4 dilution of Coomasie Brilliant Blue G-250[5] dye was added to each well. After 10 min of incubation, the samples were read in a Revelation 6.0 microplate reader[6] at 595 nm.

After protein determination, the samples were prepared for protein electrophoresis. A loading sample of 300μL with a final protein concentration of 1μg/μL was prepared using the formula V_I X C_I=V_F X C_F; where V_I = initial volume from the protein sample, C_I = concentration of the protein sample, V_F = Final volume of the loading sample (300μL), and C_F = final concentration of the loading sample (1μg/μL). For example if a protein sample had a concentration of 5.58μg/μL, then substitute in to the formula to determine the sample volume needed to prepare the sample; V_I X 5.58μg/μL = 300μL X 1μg/μL. The volume of protein sample needed to prepare the sample would be 53.8μL. After the sample volume was determined, distilled water was added to bring the sample volume to 100μL. Then 200μL of Laemmli (Laemmli, 1970) sample buffer (62.5mM Tris-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% bromophenol blue) was added to the sample to complete the 300μL of loading sample volume. Next, 10.5μL of β-mercaptoethanol was added before loading the sample. The samples were heated at 95°C for five minutes before loading, and 15μg of sample were loaded in

[5] Bio-Rad, Hercules, Ca 94547
[6] Dinex Technologies, Chantilly, Va 20151-1683

triplicate onto a Criterion Tris-HCl 10% polyacrylamide gel[5] (Laemmli, 1970). The SDS-PAGE electrophoresis was conducted using a Criterion precast gel tank[5]. The gels ran at 200V constant power, and a current of 90mA/ gel, for 55 minutes.

D. Western blot analysis for hsp70

After protein fractionation through SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane (45μm) using a mini transblot electrophoretic transfer cell[5]. Transfer was performed at 100 volts constant power and 300mA constant current for 1 hour.

The detection of proteins was done at room temperature, using the Tropix Western-Light™ chemiluminescent system[7]. After the transfer, the membrane was rinsed for 1 minute in 1X TBS buffer (20mM Tris, 500mM NaCl, pH 7.5). Then the membranes were incubated in 15mL of blocking buffer (1X TBS, 0.2% I-Block™ reagent, 0.1%Tween®-20 detergent) for 30 minutes.

A volume of 15μL of anti-hsp70 antibody[8] was added to 15mL of blocking buffer (1:1000 dilution), and incubated for 1 hour. After primary antibody incubation, the membranes were washed two times for 5 min each washing using 20mL of TTBS buffer (20mM Tris, 500mM NaCl, 0.05% Tween®-20, pH 7.5) per wash. After the washes, the membranes were incubated using 3μL of goat anti-mouse IgG + IgM alkaline phosphatase antibody conjugate[7] in 15mL of blocking buffer (1:5,000 dilution) for 1 hour. After secondary antibody incubation, the membranes were washed three times for 5

[7] Applied Biosystems, Bedford, Ma 01730
[8] Sigma, St. Louis, Mo 63118

min each washing in TTBS buffer. Following the TTBS washes, the membranes were washed two times for 2 min each washing in 1X assay buffer[7].

Afterwards, the membranes were placed on a plastic wrap and 3mL of CSPD® chemiluminescent substrate[7] substrate was added for 5 min. The membranes were drained and placed on a plastic development folder for X-ray film exposure. The membrane was placed in contact to standard X-ray film for 5 min. After the exposure, the membranes were placed in a developer solution for 5-10 sec. They were washed then in tap water for 1 min, and placed for 2 min into a fixer solution. After the fixer solution, the film was washed in tap water for 1 min and dried.

E. Hsp70 quantification

A standard curve was prepared using solutions of purified hsp70 (1.3μg/μL) [8] in Laemmli sample buffer. A volume of 1μL of hsp70 in 34.2μL of sample buffer, and 1.8μL of β-mercaptoethanol were used to make a final concentration of 0.038μg/μL of solution to load 0.8, 0.6, 0.4, 0.2, and 0.1μg on polyacrylamide gels. Another solution was made using 1μL of hsp70 in 342.1μL of sample buffer, and 18μL of β-mercaptoethanol for a final concentration of 0.0038μg/μL to load 0.05, and 0.03μg on polyacrylamide gels. The SDS-PAGE electrophoresis and Western blot analysis were performed as described above.

The protein signals were analyzed using the Gel-Doc 6000 densitometer[5]. The densities of each protein standard were set in a standard curve to determine hsp70 quantity in each X-ray film. Data were expressed in ng of hsp70/ μg of total protein.

F. Glutathione peroxidase assay sample preparation and protein determination

A volume of 2mL of ice-cold PO_4-EDTA buffer (50mM PO_4; 5mM Ethylene diaminetetraacetic acid (EDTA); 1mM Phenylmethanesulfonyl fluoride(PMSF))[8] was added to 0.5g of liver sample. The samples were kept at 4°C at all times. They were minced and washed 3 times to blood residue. The samples were homogenized with an ultra-turax homogenize[2] at 20,000 rpm. The samples were centrifuged in a Beckman model J-6B centrifuge[3] at 5,000 rpm for 30 min at 4°C. After the first centrifugation, 1mL of the sample supernatant was transferred to a 1.5mL microcentrifuge tube. The samples were centrifuged in an IEC micromax RF microcentrifuge[4] at 15,000 rpm for 30 min at 4°C. After the second centrifugation, 500 μL of sample was transferred to a 1.5mL microcentrifuge tube for total protein analysis (Bradford, 1976) as described above and glutathione peroxidase (GPx) assay.

G. Glutathione peroxidase activity assay

The glutathione peroxidase (GPx) assay measures the decrease in NADPH absorbance at A_{350nm}, which reflects the activity of GPx. An instrument blank, a kinetic blank, and a sample mixture were prepared to load into microplate wells. Each well contained 110μL of reaction mixture (30mg NADPH; 125U glutathione reductase; 200mg reduced glutathione; 8mg sodium azide) [8]. Then, 150, 135, 120μL of PO_4- EDTA buffer were added to the instrument blank, the kinetic blank, and the sample mixture, respectively. The sample mixture included 15μL of each protein sample. Finally, 15μL of 2.5mM of hydrogen peroxide were added to the kinetic blank and the sample mixture. Each component was added in the order described above. The samples were read in a

MRX Revelation TC microplate reader[6]. The instrument has a kinetics setting, which read the plate 7 times with a 30 sec interval between readings, at room temperature, at a wavelength of 340nm.

When the plate reader completed the absorbance readings, the results were used to determine GPx activity by the following formula: [(Δ sample ABS/min - Δ kinetic blank ABS/min)/ 0.00622]/ loaded protein (mg). The loaded protein is the sample volume (15μL) X its protein concentration. The optical density of 1mM solution of NADPH was 0.00622. The GPx activity was expressed as nmol of NADPH oxidized per minute per mg of total protein, which is the same as mU/mgTP.

F. Statistical analysis

The comparison of hsp70 concentrations and GPx activity among treatments was accomplished with the GLM procedure from SAS (2001) version 8.0[9]. Means were separated by least significant difference.

II. Experiment 2

A. Egg collection and incubation

The purpose of this experiment was to determine the embryonic liver hsp70 concentration and GPx activity before and after heat stress. The eggs used for this experiment were collected from commercial hatcheries. There were 100 eggs from a non-

[9] SAS Institute, Cary, N.C. 27513

Se-supplemented flock and 90 eggs from a flock supplemented with 0.3ppm of selenium in yeast[1]. The eggs were incubated at 37.5°C for 21d. At d 22, 50 eggs from the non-Se supplemented flock and 45 eggs from the Se-supplemented flock were heated at 40°C for 2 hours. After the heating episode, the eggs were returned to the incubator set at 37.5°C for 3h. The remaining 50 eggs from the non- Se-supplemented flock and 45 eggs from the Se-supplemented flock were held constantly at 37.5°C until sample collection. The sample collection was done as described in experiment 1. The hsp70 analysis and the GPx activity assay were performed as described in experiment 1.

B. Statistical analysis

Comparisons of hepatic hsp70 concentration and hepatic GPx activity were made using the General Linear Model procedure from SAS (2001) version 8.0[9]. Means were separated by least significant difference and treatment interactions were analyzed.

Results

A. Experiment 1

In Experiment 1, there were no differences ($p>0.05$) among Se treatments for hsp70 concentration (Figure 1). Table 1 and Figures 2, 3, and 4 show that there were no significant patterns in hsp70 expression in Trials 1, 2, and 3.

Experiment 1 had no significant effects ($p>0.05$) of Se supplementation for GPx activity. Table 2 and Figures 2, 3 and 4 show that there was no clear treatment-associated pattern for GPx activity.

B. Experiment 2

Observation of the hsp70 western blot analysis (Figure 5) suggested a small treatment difference with the non-Se-supplemented group expressing the more intense signal after *in ovo* embryonic heat exposure. At the normal incubation temperature (37.5°C), hsp70 expression was low in both Se treatments compared to the results in experiment 1 and after 3 h of recovery from the heating episode. Pre-stress Se-treatment-associated hsp70 concentrations (Table 3 and Figure 6) were not significantly different ($p>0.05$). After 3h of recovery from the heating episode, the non Se-supplemented group had a significantly ($p<0.05$) higher hsp70 concentration than the Se- supplemented group and pre-stress groups (Table 3 and Figure 6). The Se-supplemented group remained at a near-pre-stress level after 3h of recovery showing no significant differences when compared to the pre-stress groups ($p>0.05$). There was a significant temperature by diet interaction ($p< 0.05$) in which the temperature treatment had the greatest influence in inducing hsp70 in the non-Se-supplemented samples.

Table 4 and Figure 7 show that there are Se-dependent differences in GPx activity. At normal incubation temperature, both Se treatments had a higher GPx activity than the groups after heat exposure. The differences in GPx activity before and after heating were not significant ($p>0.05$). Before stress, the non-Se-supplemented group had significantly ($p<0.05$) lower activity than the Se-supplemented group. After stress recovery, the non-Se-supplemented group still had significantly ($p<0.05$) lower activity than the Se-supplemented group.

C. Combined Trials

Table 5 and Figure 8 show the results for hepatic hsp70 concentrations for all trials. Before heat exposure, hepatic hsp70 concentrations were less than hsp70 concentrations measured immediately after heat exposure or after three hours recovery from heat exposure. Before heat exposure, the hepatic hsp70 levels did not differ between Se treatment groups ($p>0.05$). Hepatic hsp70 concentrations increased significantly ($p <0.05$) during heat exposure in Se-supplemented embryos but not in the group from hens not given Se. The difference between no Se- and Se-supplementation hsp70 concentrations was not different (p.0.05) at the end of the heat exposure. After 3h of recovery, the non-Se-supplemented group continued to accumulate hsp70 significantly ($p<0.05$) compared to 37.5 C and 40C temperature treatments. The hepatic hsp70 concentrations in the Se-supplemented group after heat exposure and 3h recovery was significantly less ($p<0.05$) than 40C only. However, the Se-supplemented group after 40C and 3 h recovery was not different from the 37.5C pre-stress group. Table 6 and Figure 9 show GPx activity for all experiments. Before stress, GPx activity levels were

higher ($p<0.05$) than post heat exposure activities. The Se-supplemented group had significantly higher activity ($p<0.05$) than the non-Se-supplemented group. The non-Se-supplemented group GPx activity, before heat exposure, was not significantly different ($p>0.05$) from GPx activities in the non-Se-supplemented heat exposed groups. The GPx activity declined during heat exposure. Immediately after heat exposure, the non-Se-supplemented group was not significantly different ($p>0.05$) from the Se-supplemented group. At 3h of recovery from heat exposure, GPx activity had started to return toward the activity of the enzyme activity in the 37.5C groups, but only the Se-supplemented group had GPx activity that was significantly higher than the activity found in livers of the Se-supplemented 40C group.

Table1. Effect of Selenium on hsp70 Concentration in Heat Stressed 22d Turkey Embryos-Experiment 1.

Level of Selenium Supplementation	*Hsp70 concentration (ng hsp70/ μg total protein)*		
	Trial 1	Trial 2	Trial 3
Not Supplemented	6.55[a]	8.06[b]	6.47[a]
Supplemented	7.36[a]	9.97[b]	5.86[a]
SEM	7.36 ± 2.3		
R^2	0.006		

[a,b] In a column, means with no common superscript indicate significant differences ($p<0.05$).

Table 2. Effect of Selenium on GPx activity in Heat Stressed 22d Turkey Embryos-Experiment 1.

Level of Selenium supplementation	*GPx activity (nmol NADPH oxidized/min/mg total protein)*		
	Trial 1	Trial 2	Trial 3
Not supplemented	7.09[ab]	9.64[b]	4.53[a]
Supplemented	6.40[ab]	12.34[b]	8.32[a]
SEM	8.11 ± 1.1		
R^2	0.013		

[a,b] Means with no common superscript indicate significant differences ($p<0.05$).

Table 3. The Effect of Selenium on hsp70 Concentration in Heat Stressed 22d Turkey Embryos – Experiment 2.

Level of selenium supplementation	*Hsp70 concentration (ng hsp70/μg total protein)/ temperature treatment*	
	37.5°C	40°C + 3h recovery
Not supplemented	4.82[a]	10.22[b]
Supplemented	4.98[a]	6.00[a]
SEM	6.5 ± 2.0	
R^2	0.35	

[a,b] Means with no common superscript indicate significant differences ($p<0.05$).

Table 4. The Effect of Selenium on GPx Activity in Heat Stressed 22d Turkey Embryos – Experiment 2.

Level of selenium supplementation	*GPx activity (nmol NADPH oxidized/min/mg total protein)/ temperature treatment*	
	37.5°C	40°C + 3h recovery
Not supplemented	12.46[ab]	8.33[a]
Supplemented	29.54[c]	22.96[b]
SEM	14.84 ± 0.5	
R^2	0.31	

[a,b,c] Means with no common superscript indicate significant differences ($p<0.05$).

Table 5. The Effect of Selenium on hsp70 Concentration in Heat Stressed 22d Turkey Embryos – Experiments 1 and 2 combined.

Level of selenium supplementation	*Hsp70 concentration (ng hsp70/μg total protein)/ temperature treatment*		
	37.5°C	40°C	40°C + 3h recovery
Not supplemented	4.82[a]	6.93[ab]	10.22[c]
Supplemented	4.98[a]	8.05[bc]	6.00[a]

[a,b,c] Means with no common superscript indicate significant differences ($p<0.05$).

Table 6. The Effect of Selenium on GPx Activity in Heat Stressed 22d Turkey Embryos – Experiments 1 and 2 combined.

Level of selenium supplementation	*GPx activity (nmol NADPH oxidized/min/mg total protein)/ temperature treatment*		
	37.5°C	40°C	40°C + 3h recovery
Not supplemented	12.46[ab]	5.90[a]	8.32[a]
Supplemented	23.43[c]	8.68[a]	13.72[b]

[a,b,c] Means with no common superscript indicate significant differences ($p<0.05$).

Figure 1. Hsp70 analysis- Experiment 1

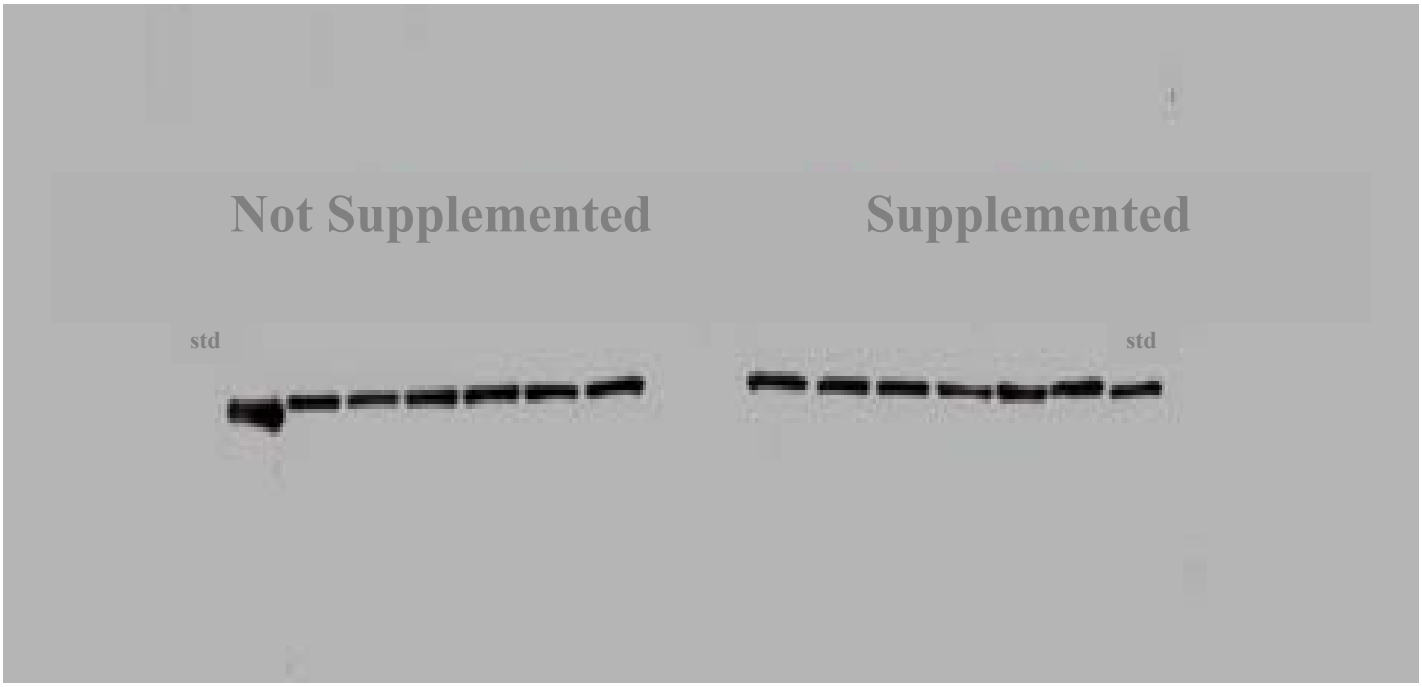


Figure 2. Comparison between hsp 70 expression and GSH-px activity- trial 1

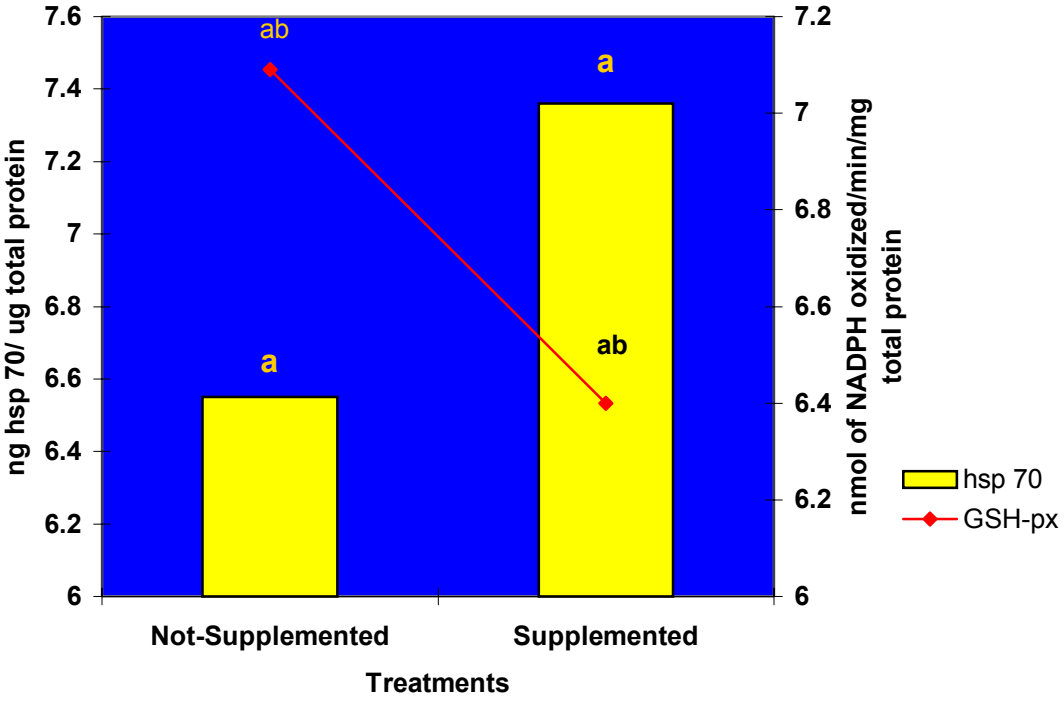


Figure 3. Comparison between hsp 70 expression and GSH-px activity- trial 2

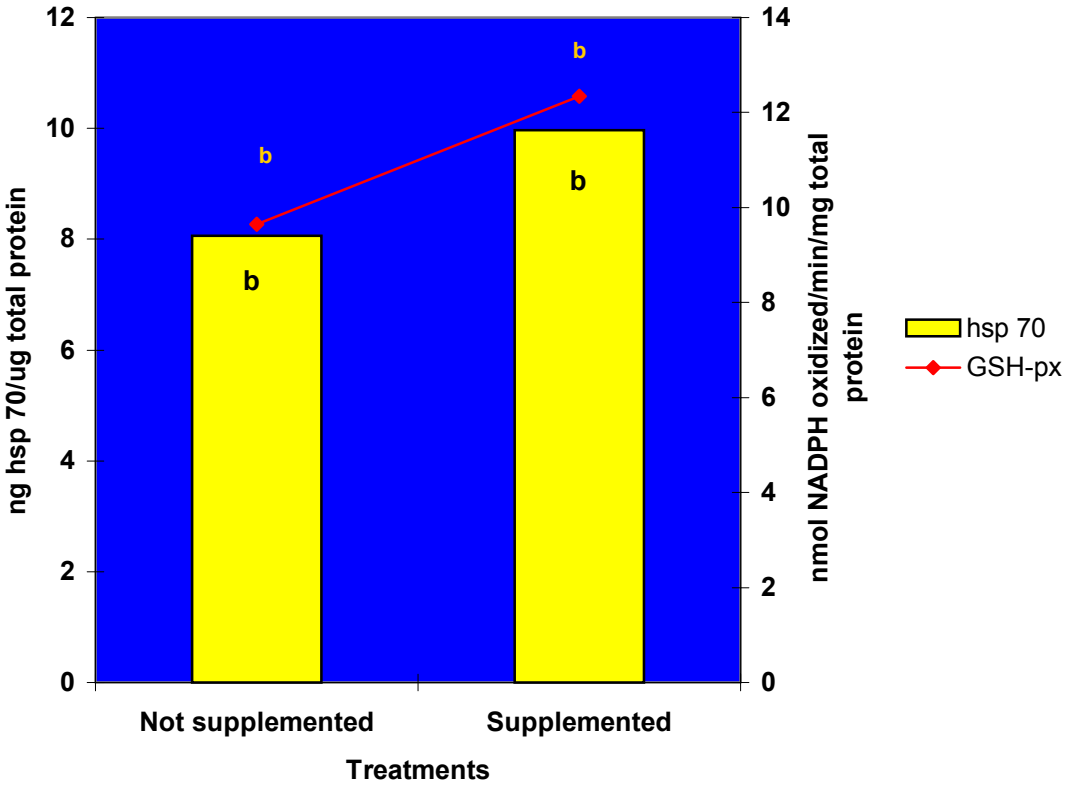


Figure 4. Comparison between hsp70 expression and GSH-px activity- trial 3

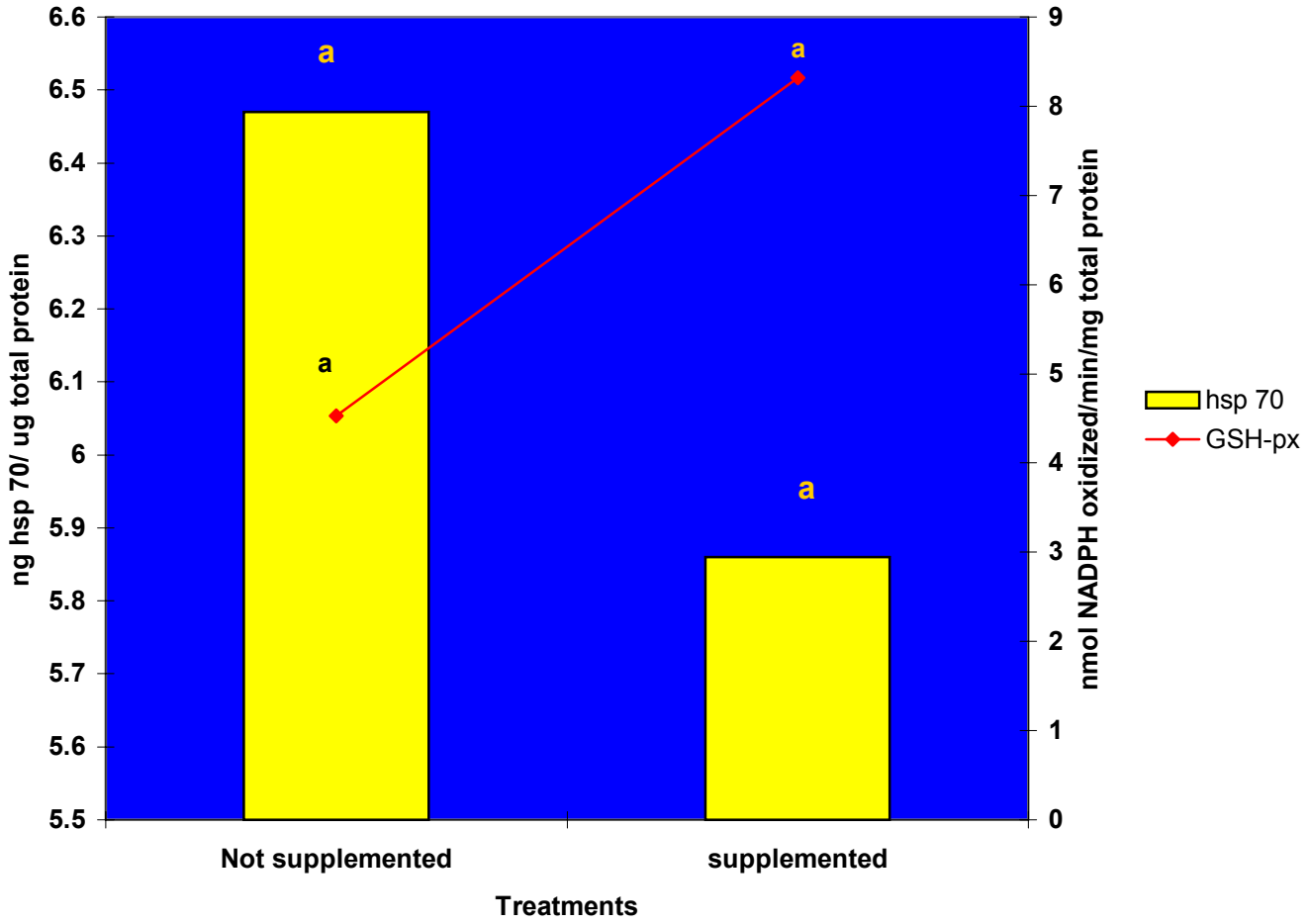


Figure 5. Hsp70 analysis- experiment 2

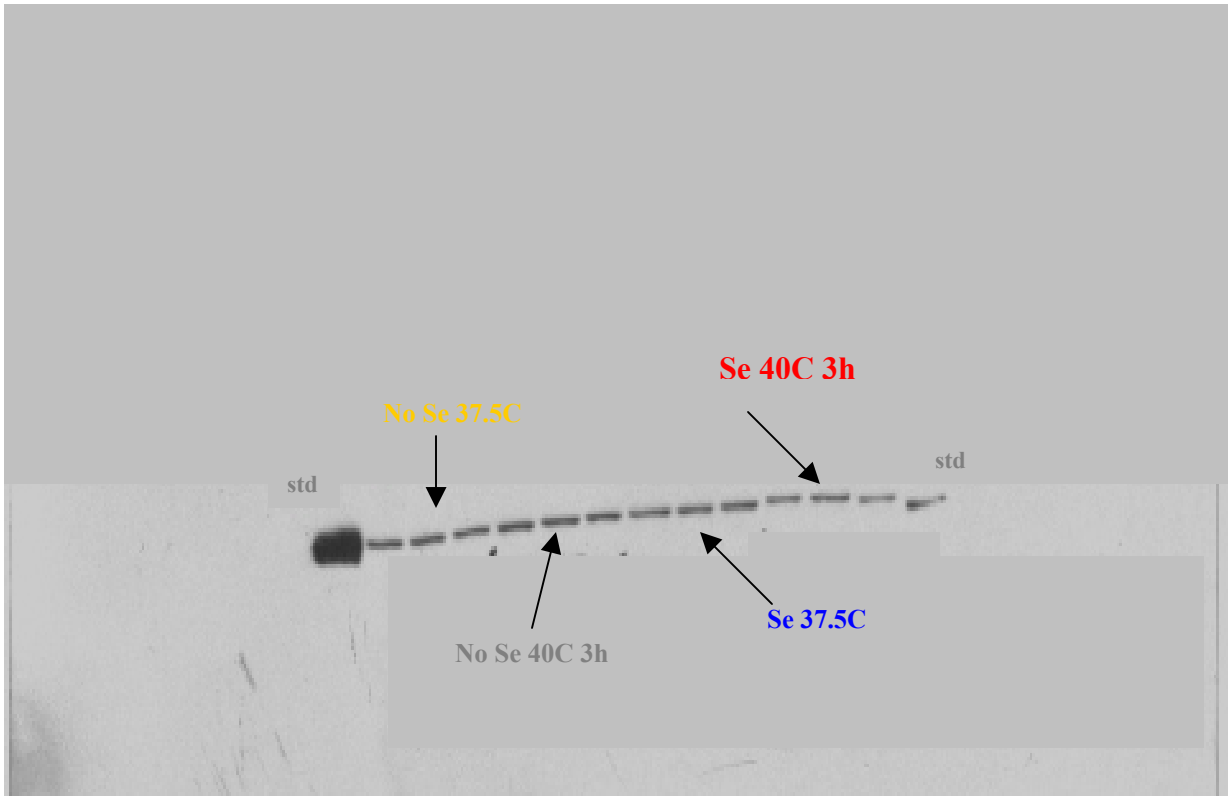


Figure 6. The effect of Selenium on hsp70 expression in heat stressed 22 day turkey embryos - experiment 2

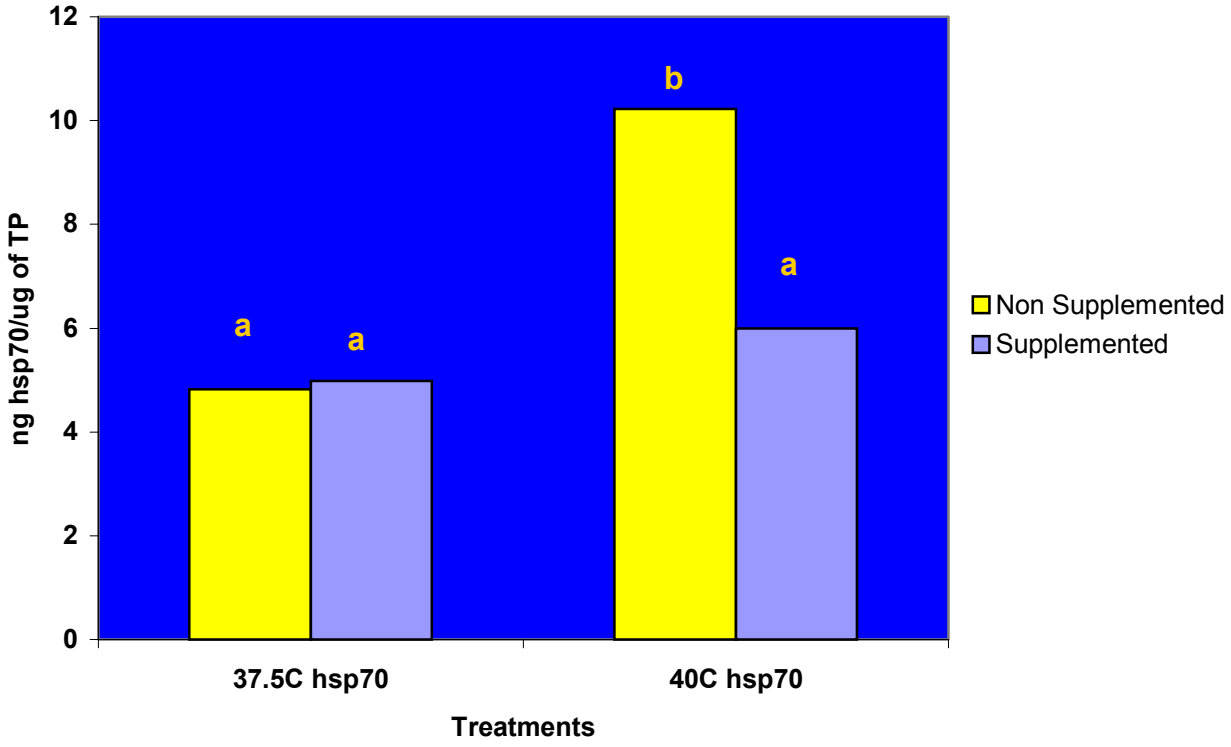


Figure 7. Glutathione Peroxidase activity in 22 day turkey embryos - experiment 2

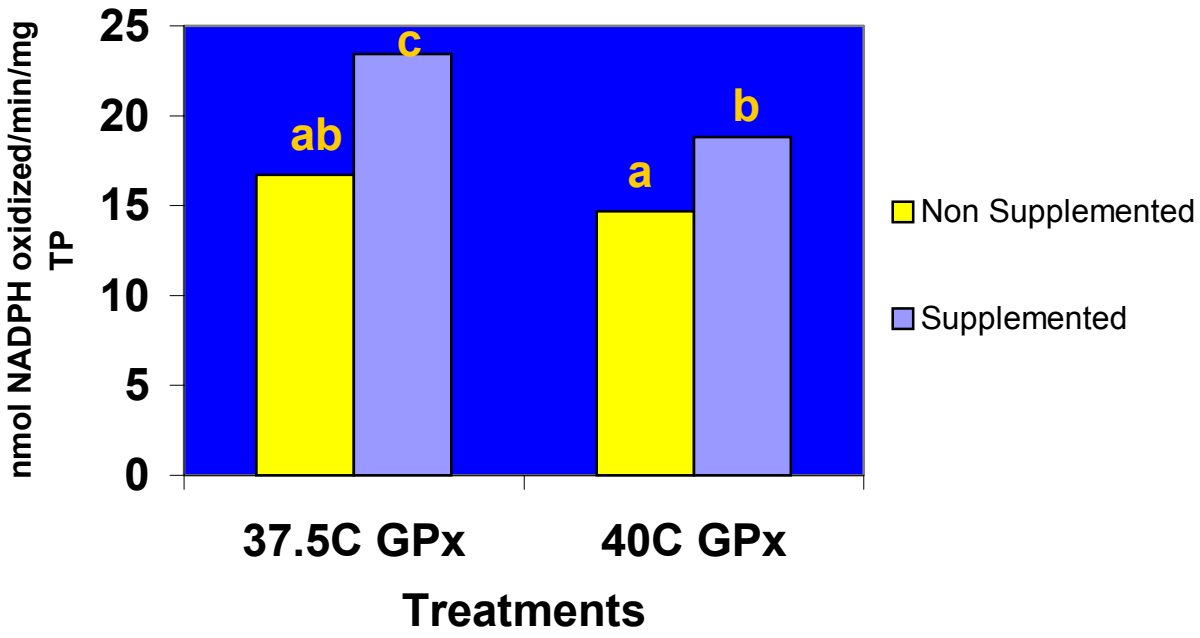


Figure 8. Hsp70 expression- combined experiments

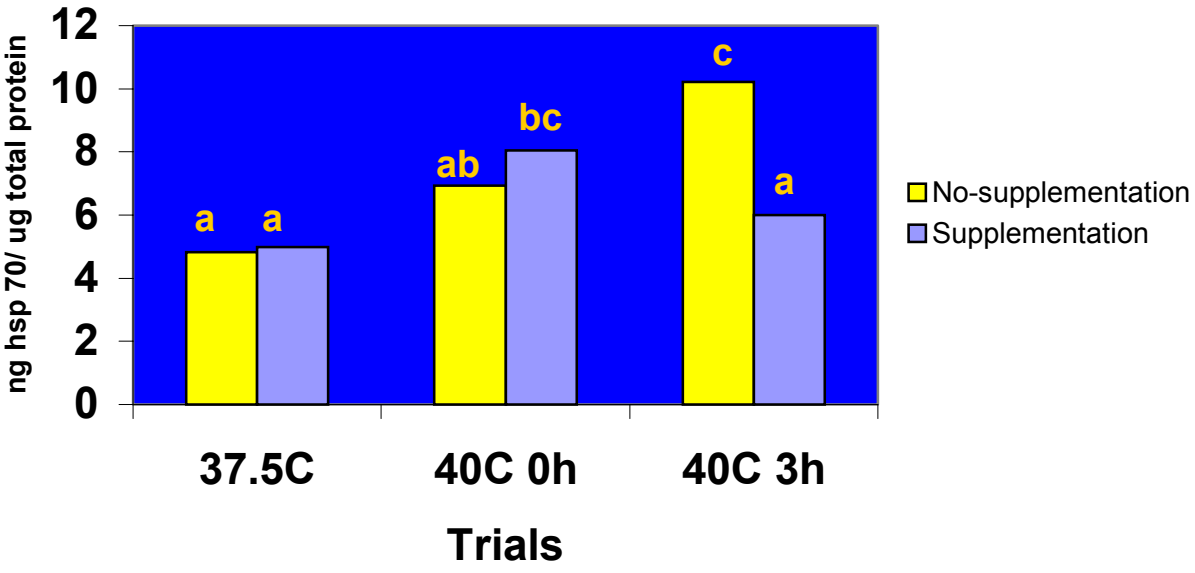
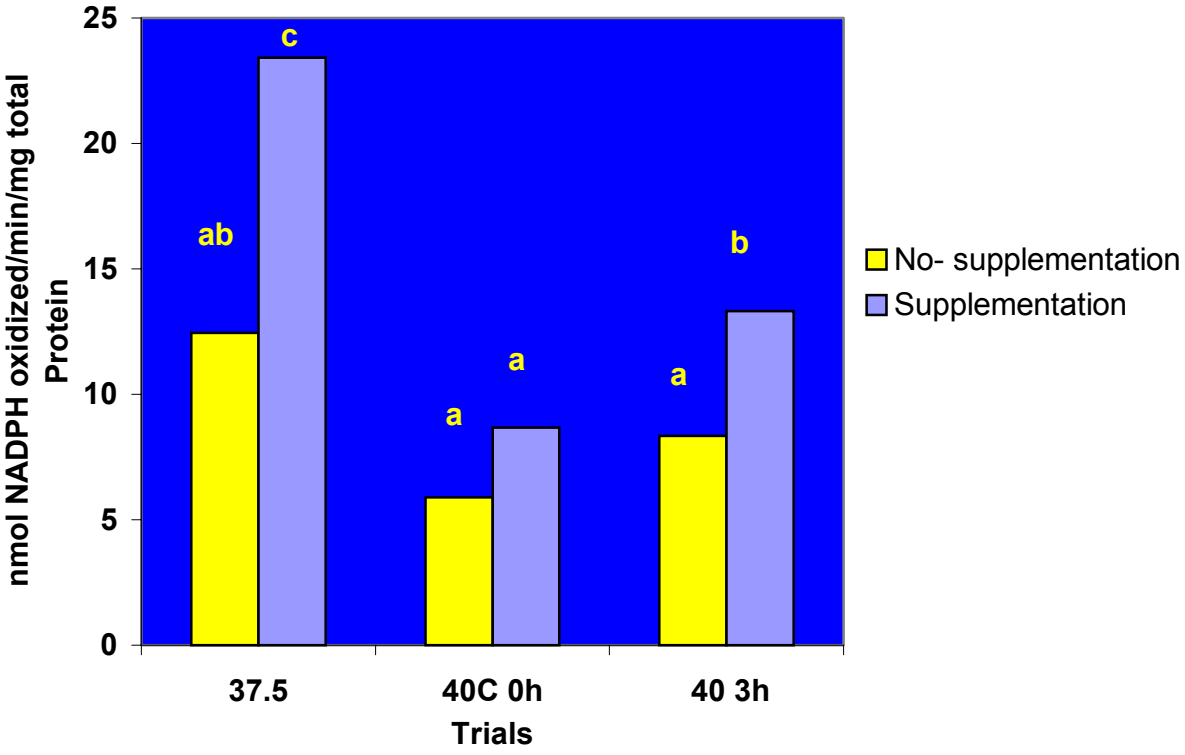


Figure 9. Glutathione Peroxidase activity- Combined trials



Discussion

Experiment 1

Neither trial 1, 2, nor 3 showed significant differences in hsp70 induction due to Se-supplementation. Givisiez et al., (2001) showed that there was hsp70 induction in heat stressed broiler chicken embryos, but induction and accumulation varied among tissues and temperature. The hsp70 concentrations may differ in tissue at different stages of development, and the basal concentrations of tissue hsp70 and hsc70 (the cognate form of hsp70) (Givisiez et al., 2001) Within various tissues you might see a variation in the intensity of the heat shock response, but a response would be seen at each embryonic stage and in each tissue. Gabriel et al. (2002) showed that hsp70 induction was dependent on incubation temperature. Embryos stressed at 41°C had non-significant increases in mRNA expression compared to non-stressed embryos, and it was significantly lower than embryos stressed at 43°C. In this study, heat might not have induced hsp70 at 40°C to a level comparable with embryos exposed to higher temperatures. Yet, if one examines the heat shock response after three hours of recovery from heat exposure (Table 5), there are clear differences in the concentrations of hsp70 that are dependent upon the Se treatment. Leandro et al. (2004) stated that a temperature of 40°C might not be appropriate to induce a heat response. Additionally, each embryo has different basal levels of protein that affected hsp70 accumulation in the liver. Leandro et al., (2004) mentioned that the organism might respond rapidly to heat stress in tissues that are more important for the normal functioning of the body. Thus, the liver might be more important in the recovery rather than during heat stress, explaining the reason why liver hsp70 accumulated slowly during recovery of embryos from dams on a diet that was not supplemented with Se and a

small but rapid increase followed by a decrease in the Se-supplemented embryos. A relationship between hsp70 expression and Se was not evident in experiment 1 because there was high variation among treatment means among trials (Table 1). In order to establish that Se-supplementation increased Se concentration in the liver; glutathione peroxidase (GPx) activity was measured

Rotruck et al. (1973) found that Se was an integral part of GPx. Cantor and Scott (1974) also reported that selenium (Se) is an integral part of the enzyme glutathione peroxidase (GPx) and found that selenium deficiency resulted in a marked drop of GPx in the liver of turkeys and chickens. and studied its role. Surai et al. (1997) showed that there are tissue specific differences in GPx activity within the chick embryo during development with the liver having the highest activity. GPx activity did not have a significant difference due to Se supplementation. Paton et al. (2002a) reported that in periods of selenium deficiency, the provision of supplemental selenium was associated with increased GPx activity. In this experiment, overall GPx activity (Table 6) was higher in Se-supplemented samples than in non-Se supplemented samples although it was not significant due to high variation in between treatment samples (Table 2). Trial 1 had lower, but not significant GPx activity in the Se supplemented samples compared to the non-Se-supplemented samples. GPx activity increases in the Se-supplemented samples are seen in trial 2 and trial 3 compared to the non-Se-supplemented samples. Arnold et al. (1972) showed that within 12 days of feeding supplemental selenium to single combed white Leghorns, the selenium content of the eggs had reached a maximum of about 1.7p.p.m. Thus, it was speculated that turkey hens, due to their larger body size, might require a longer time to incorporate more selenium into their eggs, which was suggested

by differences in GPx activity increases after each of trials 1, 2 and 3, respectively. The levels of GPx activity did not show that it influenced hsp70 expression.

Concluding from these studies, it was obvious that hsp70 expression and GPx activity in the liver should also be measured before and after stress to establish an effect between Se supplementation and heat stress. Gabriel et al. (2002) showed that there was a higher accumulation of hsp70 mRNA during recovery from heat exposure. The embryos stressed at 41°C had returned to basal levels after 3h of recovery while the embryos stressed at 43°C returned to basal levels after 6h of recovery (Gabriel et al., 2002). Padmaja et al. (1993) showed that during Se-induced lipid peroxidation in the heart muscle of chicken embryos, there was not an enhancement of activity in GPx activity. After 48 h of recovery, a 52% increase in GPx activity was observed. It seems that higher Se concentration in tissues help increase GPx activity, which in turn, protects membranes from oxidative damage. Therefore if there is reduced cell damage from oxidative stress, hsp70 does not need to accumulate for longer periods of time**.** Abe et al. (1994) found that by depleting glutathione (GSH), hsp70 increased after exposure to superoxide and hydrogen peroxide. That study also found that a 4-fold increase in GSH levels was enough to reduce hsp70 levels but not inhibit it completely. Van Remmen et al. found similar results and concluded that the increased expression of the hsp70 genes would be predicted to provide the hepatocytes with increased protection against a variety of stresses. However, the decreased expression of catalase and GPx in cultured hepatocytes would lead to an increase in the sensitivity of the hepatocytes to compounds that generate reactive oxygen species as compared to the liver *in vivo*.

Experiment 2

The embryonic hepatic hsp70 concentrations at 37° C were low compared to the hepatic hsp70 three hours after heat exposure (Table 4 and Figure 6). After 3h recovery period, the non-Se-supplemented group had higher hsp70 concentrations compared to the Se-supplemented group, which had returned to near preheat concentrations. This suggested that heat exposure had disrupted cellular integrity that required hsp70 in the process of reestablishing cellular homeostasis. Edens et al. (2001) reported that expression of hsp70 is a classical sign of stress in animals and is the physical manifestation of specific genes that are induced by stressors. However, there is a cost for this response, and it is paid in reduced growth due to a lower rate of synthesis of structural proteins in chronically stressed animals (Edens et al., 2001). Selenium (Se) supplementation helped to reduce this effect. That study showed that broilers given diets without supplemental Se had higher constitutive levels of hsp70 than those given organic Se (Edens et al., 2001). This study showed that embryos given organic Se does not need for prolonged protection from hsp70 because it keeps a higher integrity of the antioxidant defense system. The fact that there is a more reduced redox status when organic selenium is fed creates a condition within the cell that does not require additional hsp70 for its chaperone and protein folding functions.

Whelan and Hightower (1985) observed that hsp classes are induced by the oxidation of sulfhydryls. Abe et al. (1994) showed that the induction of hsp70 by cadmium was significantly enhanced after depletion of cellular glutathione (GSH). GSH depletion does not make mouse embryos more susceptible to heat shock, but the embryo does require the capacity to synthesize GSH for induced thermotolerance (Aréchiga and

Hansen, 1988). GPx activity may influence in the maintenance of GSH by affecting its redox ratio.

GPx activity was highest in Se-fed birds (Mahmoud and Edens, 2003). These effects were similar to the present study (Table 5 and Figure 7) showing that there is a greater antioxidant status that is efficient in removing free radicals from the cell. Edens et al (2001) reported that Se-fed broilers catalyzed a more rapid oxidation of GSH to GSSH because of higher glutathione peroxidase activity, and also caused increased GR activity for the reduction of GSSG back to GSH. The glutathione redox cycle must be considered because hsp70 expression is responsive to increased thiol oxidation (GSSG production) and loss of cellular GSH as indicated by a lower R:O ration (Edens et al., 2001). The removal of ROS is dependent on the oxidation of GSH to GSSG, which is catalyzed by glutathione peroxidase, a Se-dependent enzyme (Rotruck et al., 1973). In this experiment GPx activity was higher in the Se-supplemented groups compared to the non-Se-supplemented groups in both temperature treatments. GPx levels before stress were higher than after stress recovery. Mahmoud and Edens (2003) showed that there is a decrease in liver GSH levels, which was related to rapid oxidation of GSH to GSSG during the antioxidation process induced by heat distress. Sel-Plex®-feeding effectively altered the antioxidant status of heat stressed broilers (Mahmoud and Edens, 2003). The improved redox status of heat distressed broiler chickens was attributed to higher GPx activity in Sel-Plex®-fed birds (Mahmoud and Edens, 2003).

In this experiment, low concentrations of hsp70 before heat stress was associated with higher GPx activity in embryos from dams fed Sel-Plex®. After stress, a lower GPx activity in the non-Se-supplemented group was reflected by higher hsp70 expression. A

higher GPx activity in the Se-supplemented group did not promote continued high concentrations of hsp70. With supplemented organic selenium, glutathione peroxidase will facilitate faster removal of metabolic oxidants from the cell and as a result less hsp70 will be required to aid in cellular protein repair caused by uncontrolled oxidative products. Thus, recovery of protein production in the cell, especially of other enzymes such as glutathione reductase (GR) and Glucose-6-Phosphodehydrogenase (G6PDH), which interact to inactivate ROS. GR will reduce GSSG back to GSH and G6PDH helps in the production of NADPH from glucose through the pentose pathway (Mahmoud and Edens, 2003).

Combined Trials

Both experiments were combined to compare the results before, during, and after stress. Before stress, hsp70 levels were low compared to the other temperature treatments (Table 5 and Figure 8). Using the means from all the trials in experiment 1, there was an induction of hsp70, although not significant for the non-Se-supplemented group. The hsp70 levels from the Se-supplemented group were significantly higher than pre-stress levels, which it can be speculated that Se supplementation causes a higher expression during stress, but during stress recovery, hsp70 concentrations increased significantly as compared with Se-supplemented embryos (Table 5). The data suggest that selenium supplementation helped hsp70 peak at lower levels, and allowed for faster recovery from stress.

When all experiments were combined, GPx activity was the highest before stress (Table 6 and Figure 9). GPx activity in both selenium treatments decreased significantly during stress, but after recovery, the Se-supplemented group had a faster recovery of activity. The Se-supplemented group had the highest activity among all temperature treatments. During stress a decline GPx activity is associated with induction of hsp70. Mahmoud and Edens (2003) showed that during stress, there is an efflux of GSH from the liver to the blood stream, decreasing GSH levels and GPx activity in the liver. This triggered an elevation in liver hsp70 synthesis in basal selenium chickens compared to selenium-fed birds. The decline in GPx activity may allow an increase in ROS resulting from thiol oxidation (Whelan and Hightower, 1985). In this study, an increase in GPx activity in the Se-supplemented group is associated with the decline in hsp70 expression, after stress recovery; while a slower recovery of GPx activity in the non-Se-supplemented group allowed for a prolonged expression of hsp70.

In conclusion, it is important to have a high antioxidant status to remove ROS from the cell because induction of hsp70 is an acute phase response that cannot be tolerated for long periods of time without resulting in the slowing of growth and performance in animals (Edens et al., 2001). This decreasing growth rate causes many abnormalities in hatching embryos. The hsp70 family of proteins functions in the protection and folding of newly synthesized protein and the refolding of recently denatured proteins (Lindquist and Craig, 1988). After stress recovery, hsp70 helps to establish normal protein synthesis in the liver allowing an increase in GSH concentrations and GPx activity. This process can be accelerated by having higher antioxidant integrity in the cell before stress, which helps the embryo overcome the toxic effects of induced

ROS. Thus, higher cellular integrity can be achieved due to improved availability of certain nutrients such as organic selenium, and this ultimately will facilitate molecular mechanisms that will benefit animal health.

REFERENCES

Abe, T., Konishi, T., Katoh, T., Hirano, H., Matsukuma, K., Kashimura, M., and Higashi, K., 1994. Induction of heat shock 70 mRNA by cadmium is mediated by glutathione suppressive and non-suppressive triggers. Biochim. et Biophys. Acta 1201:29-36.

Al-Katanani, Y.M. and Hansen, P.J., 2002. Induced thermotolerance in bovine two-cell embryos and the role of heat shock protein 70 in embryonic development. Molec. Reprod. And Develop. 62:174-180.

Al- Saffar, A.A. and Rose, S.P, 2002.ambient temperature and the egg laying characteristics of laying fowl. World's Poult. Sci. 58:317-331.

Altan, O., Pabuccuoglu, A., Altan, A., Konyalioglu, S., and Bayraktar, H., 2003. Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. Br. Poult. Sci. 44:545-550.

Ande, T.B. and Wilson, H.R., 1981. Hatchability of chicken embryos exposed to acute high temperature stress at various ages. Poult. Sci. 60:1531-1566.

Ando, M., Katagiri, K., Yamamoto, S., Asanuma, S., Usuda, M., Kawahara, I., and Wakamatsu, K., 1994. Effect of hyperthermia on glutathione peroxidase and lipid peroxidative damage in liver. J. Therm. Biol. 19:177-185.

Aréchiga, C.F. and Hansen, P.J., 1988. Response of preimplantantation murine embryos to heat shock as modified by developmental stage and glutathione status. In Vitro Cell. Dev. Biol.- Animal 34:655-659.

Arnold, R.L., Olson, O.E., and Carlson, C.W., 1972. Selenium withdrawal and egg selenium content. Poult. Sci. 51:341-342.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.

Cantor, A.H. and Scott, M.L., 1974. The effect of selenium in the hen's diet on egg production, hatchability, performance of progeny and selenium concentration in eggs. Poult. Sci. 53:1870-1880.

Christensen, V.L., Davis, G.S., and Nestor, K.E., 2002. Environmental incubation factors influence embryonic thyroid hormones. Poult. Sci. 81:442-450.

Combs, G.F. and Scott, M.L., 1979. The selenium needs of laying and breeding hens. Poult. Sci. 58:871-884.

Decuypere, E. and Michels, H., 1992. Incubation temperature as a management tool: A review. W. Poult. Sci. 48:28-38.

Diplock, A.T., 1976. Metabolic aspects of selenium action and toxicity. CRC Crit. Rev. in Tox. 4:271-329.

Edens, F.W., 2001. Involvement of Sel-Plex in physisological stability and performance of broiler chickens. Pages 349- 376 in: Biotechnology in the feed industry. Proceedings of Alltech's 17th annual symposium. T.P. Lyons and K.A. Jacques (eds.), Nottingham University Press, Nottingham, U.K.

Edens, F.W., Givisiez, P.E.N., Mahmoud, K.Z., and Sefton, A.E., 2001. Influence of organic selenium on hsp70 response of heat stressed and enteropathogenic *Escherichia coli*- challenged broiler chickens. Pages 64-67 In: Proceedings for the 50th Western Poultry Disease Conference, University of California, Davis Ca.

Edens, F.W., 2002. Practical applications for selenomethionine: broiler breeder reproduction. Pages 29- 42 in: Nutritional biotechnology in the feed and food industry. Proceedings of Alltech's 18th annual symposium. T.P. Lyons and K.A. Jacques (eds.), Nottingham University Press, Nottingham, U.K.

Edwards, J.L., King, W.A., Kawarsky, S.J., and Ealy, A.D., 2001. Responsiveness of early embryos to environmental insults: potential protective roles of hsp70 and glutathione. Theriogenology 55:209-223.

Ellis, R.J., 1996. Chaperonins: introductory perspective. Pages 2-25 in: The Chaperonins. R. J. Ellis (editor). Academic Press, San Diego, Ca.

Fitzsimmons, R.C. and Phalaraksh, K., 1978. Chick embryonic development as influenced by *in ovo* injected selenium. Can. J. Anim. Sci. 58:227-232.

Flohé, R.B., 1999.Tissue-specific functions of individual glutathione peroxidases. Fr. Rad. Biol. Med. 27:951-965.

Foresta, C., Flohé, L., Garolla, A., Roven, A., Ursini, F., and Maiorino, M., 2002. Male fertility is linked to the selenoprotein PHGPx. Biol. of Rep. 67:967-971.

French, N., 1986. Recent developments in the hatchability of turkey eggs. Turkeys 34 (3): 25-27.

French, N., 1994. Efect of incubation temperature on the gross pathology and development of turkey embryos. Br. Poult. Sci. 35:363-371.

French, N.A., 1997a. Modeling incubation temperature: The effects of incubator design, embryonic development, and egg size. Poult Sci. 76:124-133.

French, N.A., 1997b. Overheating turkey eggs: Critical periods during incubation. proceedings of the 10th European Poultry Conference, Jerusalem, Israel. 2:790-794.

Gabriel, J.E., da Mota, A.F., Boleli, I.C., Macari, M., and Coutinho, L.L., 2002. Effect of moderate and severe heat stress on avian embryonic hsp70 gene expression. Growth, Develop., and Aging 66:27-33.

Gabriel, J.E., Alvares, L.E., Gobet, M.C., de Paz, C.C.P., Packer, I.U., Macari, M., and Coutinho, L.L., 2003. Expression of myoD, myogenin, myostatin and hsp70 transcripts in chicken embryos submitted to mild cold or heat. J. Therm. Biol. 28:261-269.

Givisiez, P.E.N., da Silva, M.M., Mazzi, C.M., Ferro, M.I.T., Ferro, J.A., Gonzales, E., and Macari, M., 2001. Heat or cold chronic stress affects organ weights and hsp70 levels in chicken embryos. Can. J. An. Sci. 81:83-87.

Gregory III, J.F., 1984. Effect of dietary selenium on the metabolism of aflatoxin B_1 in turkeys. Fd. Chem. Toxic. 22:637-642.

Hahn, G.M. and Li, G.C., 1990. Thermotolerance, thermoresistance, and thermosensitisation. Pages 79-100 in: Stress proteins in biology and medicine. R.I. Morimoto, A. Tissieres, and C. Georgopoulos (eds.), Cold Spring Harbor Laboratory Press, New York, N.Y.

Heydari, A.R., You, S., Takahashi, R., Gutsmann, A., Sarge, K.D., and Richardson, A., 1996. Effect of caloric restriction on the expression of heat shock protein 70 and the activation of heat shock transcription factor 1. Develop.Gen.18:114-124.

Heywang, B.W., 1944. Fertility and hatchability when the environmental temperature of chickens is high. Poult. Sci. 23:334-339.

Hightower, L.E., 1993. A brief perspective on the heat-shock response and stress proteins. Marine Environ. Res. 35:79-83.

Hightower, L.E., Sadis, S.E., and Takenaka, I.M., 1994. Interactions of vertebrate hsc70 and hsp70 with unfolded proteins and peptides. Pages 179-207 in: The biology of heat shock proteins and molecular chaperones. R.I. Morimoto, A. Tissieres, C. Georgopoulos (eds.) Cold Spring Harbor Laboratory Press, New York, N.Y.

Hightower, L.E. and Leung, S., 1997. Mammalian hsc70 and hsp70 proteins. Pages 53-58 in: Guidebook to molecular chaperones and protein folding catalysts. M. J. Gething (editor). Oxford, University Press, Oxford, U.K.

Hoshida, S., Aoki, K., Nishida, M., Yamashita, N., Igarashi, J., Hori, M., Kuzuya, T., and Tada, M., 1997. Effects of pre-conditioning with ebselen on glutathione metabolism and stress protein expression. J. Pharm. and Exp. Ther. 281:1471-1475.

Jensen, L.S., Colnago, G.L., Takahashi, K., and Akiba, Y., 1986. Dietary selenium status and plasma thyroid hormones in chicks. Biol. Trace Element Res. 10:11-18

Johnston, R.N. and Kucey, B.L., 1988. Competitive inhibition of hsp70 gene expression causes thermosensitivity. Science 242:1551-1554.

King, Y., Lin, C., Lin, J., and Lee, W., 2002. Whole-body hyperthermia-induced thermotolerance is associated with the induction of heat shock protein 70 in mice. J.Exp. Biol. 205:273-278.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 226:112-115.

Langer, T. and Neupert, W., 1996. Chaperonin-mediated folding and assembly of proteins in mitochondria. Pages 91- 106 in: The chaperonins. R. J. Ellis (editor). Academic Press, San Diego, Ca.

Latshaw, J.D. and Biggert, M.D., 1981. Incorporation of selenium into egg proteins after feeding selenomethionine or sodium selenite. Poult. Sci. 60:1309-1313.

Latshaw, J.D. and Osman, M., 1974. Distribution of selenium in egg white and yolk after feeding natural and synthetic selenium compounds. Poultry Sci. 54:1244-1252.

Leandro, N.S.M., Gonzales, E., Ferro, J.A., Ferro, M.I.T., Givisiez, P.E.N., and Macari, M., 2004. Expression of heat shock protein in broiler embryo tissues after acute cold or heat stress. Molec. Reprod. And Develop. 67:172-177.

Lindquist, S., 1986. The heat-shock response. Ann. Rev. Biochem. 55:1151-1191.

Lindquist, S. and Craig, E.A., 1988. The heat shock proteins. Ann. Rev. Genet. 22:631-77.

Magat, W. and Sell, J.L., 1979. Distribution of mercury and selenium in egg components and egg-white proteins. Proc. Soc. Exp. Biol. Med. 161:458-463.

Mahmoud, K., 2000. Genetic and environmental variations of chicken heat shock protein. PhD. Diss., North Carolina State University, Raleigh, N.C.

Mahmoud, K.Z. and Edens, F.W., 2003. Influence of selenium sources on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (*Gallus dmesticus*). Comp. Biochem. And Phys.- Biochem and Molec. Biol. 137:921-934.

Mathiu, P.M., Whittow, G.C., and Dawson, W.R., 1992. Hatching and the establishment of thermoregulation in the wedge-tailed shearwater (*Puffinus pacificus*). Phys. Zool. 65:583-603.

McDaniel, C.D., Bramwell, R.K., Wilson, J.L., and Howarth, B., 1995. Fertility of male and female broiler breeders following exposure to elevated ambient temperatures. Poult. Sci. 74:1029-1038.

McGowan, C. and Donaldson, W.E., 1987. Lead effects in the chick during selenium deficiency. Comp. Biochem. Physiol. 88 (C):23-25.

Meijerhof R. and Albers, G., 1998. The influence of incubation conditions on the incidence of ascites. Incubation and fertility research group meeting: Abstracts. 119-120.

Mezquita, B., Mezquita, C., Mezquita, J., 1998. Marked differences between avian and mammalian testicular cells in the heat shock induction and polyadenilation of hsp70 and ubiquitin transcripts. FEBS Letters 436:382-386.

Mirkes, P.E., Cornel, L.M., Wilson, K.L., and Dilmann, W.H., 1999. Heat shock protein 70 (hsp70) protects postimplantation murine embryos from the embryolethal effects of hyperthermia. Develop. Dyn. 214:159-170.

Moraes, V.M.B., Malheiros, R.D., Bruggeman, V., Collin, A., Tona, K., Van As, P., Onagbesan, O.M., Buyse, J., Decuypere, E., and Macari, M., 2003. Effect of thermal conditioning during embryonic development on aspects of physiological responses of broilers to heat stress. J. Therm. Biol. 28:133-140.

Nichelmann, M., Burmeister, A., Janke, O., Hochel, J., and Tzschentke, B., 1998. Avian embryonic thermoregulation: Role of Q_{10} in interpretation of endothermic reactions. J. Therm. Biol. 23:369-376.

Nichelmann, M. and Tzschentke, B., 1999. Thermoregulation in precocial avian embryos. Ornis Fennica 76:177-187.

Nichelmann, M., Janke, O., and Tzschentke, 2001. Efficiency of thermoregulation in precocial avian species during the prenatal period. J. Therm. Biol. 26:273-280.

Norry, F.M. and Loeschcke, V., 2003. Heat-induced of a molecular chaperone decreases by selecting for long-lived individuals. Exp. Geron. 38:673-681.

Padmaja, K., Somasekharaiah, B.V., and Prasad, A.R.K., 1993. Selenium induced lipid peroxidation in heart tissues of chick embryos. Bull. Environ. Contam. Toxicol. 51:401-408.

Padmaja, K., Somasekharaiah, B.V., and Prasad, A.R.K., 1997. Inhibition of lipid peroxidation by selenium in chick embryos. Drug and Chem. Tox. 20:79-98.

Palmer, I.S., Arnold, R.L., and Carlson, C.W., 1973. Toxicity of various selenium derivatives to chick embryos. Poultry Sci. 52:1841-1846.

Paton, N.D., Cantor, A.H., Pescatore, A.J., Ford, M.J., and Smith, C.A., 2000. Effect of dietary selenium source and level of inclusion on selenium content of incubated eggs. Poult. Sci 79 (Suppl. 1):40.

Paton, N.D., Cantor, A.H., Pescatore, A.J., Ford, M.J., and Smith, C.A., 2002. absorption of selenium by developing chick embryos during incubation. Pages 107-121 in: Nutritional biotechnology in the feed and food industry. Proceedings of Alltech's 18th Annual Symposium. T.P. Lyons and K.A. Jacques (eds.), Nottingham University Press, Nottingham, U.K

Paton, N.D., Cantor, A.H., Pescatore, A.J., Ford, M.J., and Smith, C.A., 2002. The effect of dietary selenium source and level on the uptake of selenium by developing chick embryos. Poult. Sci. 81:1548-1554.

Pelham, H.R.B., 1990. Functions of the hsp70 protein family: An overview. Pages 287-299 in: Stress proteins in biology and medicine. R.I. Morimoto, A. Tissieres, and C. Georgopoulos (eds.), Cold Spring Harbor Laboratory Press, New York, N.Y.

Petruk, A., Korver, D.R., and Zuidhof, M.J., 2000. Effect of heat stress and age of increasing dietary Ca on reproductive performance of broiler breeder hens. Poult. Sci 79 (Suppl. 1):42.

Romanoff, A.L., Smith, L.L., and Sullivan, R.A., 1938. Biochemistry and biophysics of the developing hen's egg. 3. Influence of temperature. Memorandum of Cornell University Ag. Exp. Sta. 216:1-42.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G., 1973. Selenium: Biochemical role as a component of glutathione peroxidase. Science 179:588-590.

Rutz, F., Pan, E.A., Xavier, G.B., Anciuti, M.A., 2003. Meeting selenium demands of modern poultry: Responses to Sel-Plex™ organic selenium in broiler and breeder diets. Pages 147- 161 in: Nutritional biotechnology in the feed and food industry. Proceedings of Alltech's 19th annual symposium. T.P. Lyons and K.A. Jacques (eds.), Nottingham University Press, Nottingham, U.K.

Schlesinger, M.J., 1985. Stress response in avian cells. Pages 183-195 in: Changes in eukaryotic gene expression in response to environmental stress. B.G. Atkinson and D.B. Walden (eds.). Academic Press, inc., Orlando, Fl.

Schlesinger, M.J., 1986. Heat Shock Proteins: The search for functions. J. of Cell. Biol. 103:321-325.

Schlesinger , M.J., Collier, N.C., Agell, N., and Bond, U., 1989. Molecular events in avian cells stressed by heat shock and arsenite. Pages 137-148 in: Stress-induced proteins, Proceedings of a Hoffman-LaRoche- director's sponsors-UCLA symposium, Keystone, Colorado. Alan R. Liss, Inc. New York, N.Y.

Shallom J.M., Di Carlo, A.L., Ko, D., Penafiel, L.M., Nakai, A., and Litovitz, T.A., 2002. Microwave exposure induces hsp70 and confers protection against hypoxia in chick embryos. J. Cell. Biochem. 86:490-496.

Southgate, R., Mirault, M.E., Ayme, A., and Tissieres, A., 1985. Organization, sequences, and induction of heat shock genes. Pages 3-30 in: Changes in eukaryotic gene expression in response to environmental stress. B.G. Atkinson and D.B. Walden (eds.). Academic Press, inc., Orlando, Fl.

Surai, P.F., Speake, B.K., Noble, R.C., and Sparks, N.H.C., 1997. Antioxidant systems of the developing chicken embryo: Glutathione peroxidase. Br. Poult Sci. 38(Suppl. 1):519-520.

Surai, P.F., 1999. Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. Br. Poult. Sci. 40:397-405.

Surai, P.F., 2000. Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. Br. Poult. Sci. 41:235-243.

Surai, P.F., 2002. Selenium in poultry nutrition 1. Antioxidant properties, deficiency and toxicity. W. Poult. Sci. J. 58:333- 346.

Swann, G.S. and Brake, J., 1990. Effect of dry-bulb temperature, relative humidity, and eggshell conductance during days 17 to 21 of incubation on egg weight loss and chick weight. Poult. Sci. 69:545-553.

Van Brecht, A., Aerts, J.M., Degraeve, P., and Berckmans, D., 2003. Quantification and control of the spatiotemporal gradients of air speed and air temperature in an incubator. Poult. Sci. 82:1677-1687.

Van Remmen, H., Williams, M.D., Heydari, A.R., Takahashi, R., Chung, H.Y., Yu, B.P., and Richardson, A., 1996. Expression of genes coding for antioxidant enzymes and heat shock proteins is altered in primary cultures of rat hepatocytes. J. Cell. Phys. 166:453-460.

Voellmy, R. and Bromley, P.A., 1982. Massive heat-shock polypeptide synthesis in late chicken embryos: Convenient system for study of protein synthesis in highly differentiated organisms. Molec. And Cell. Biol. 2:479-483.

Wang, S. and Edens, F.W., 1994. Hsp70 mRNA expression in heat-stressed chickens. Comp. Biochem. Physiol. 107 (B):33-37.

Wang, S. and Edens, F.W., 1998. Heat conditioning induces heat shock proteins in broiler chickens and turkey poults. Poult. Sci. 77:1636-1645.

Weytjens, S., Meijerhof, R., Buyse, J., and Decuypere, E., 1999. Thermoregulation in chicks originating from breeder flocks of two different ages. J. Appl. Poult. Res. 8:139-145.

Wheelan, S.A. and Hightower, L.E., 1985. Differential induction of glucose-regulated and heat shock proteins: Effects of pH and sulfhydryl-reducing agents on chicken embryo cells. J. Cell. Phys. 125:251-258.

Wilson, H.R., 1991. Physiological requirements of the developing embryo: Temperature and turning. Page 145-156 in: Avian incubation- poultry science symposium num. 22. S.G. Tullet (editor). Buterworths-Heinnemann, London, U.K.

Yahav, S., Shamay, A., Horev, G., Bar-Ilan, D., Genina, O., and Friedman-Einat, M., 1997. Effect of acquisition of improvement thermotolerance on the induction of heat shock proteins in broiler chickens. Poult. Sci. 76:1428- 1434.

Yalcin, S. and Siegel, P.B., 2003. Exposure to cold or heat during incubation on developmental stability of broiler embryos. Poult. Sci. 82:1388-1392.

Zdunczyk, Z., Jankowski, J., and Koncicki, A., 2002. Growth performance and physiological state of turkeys fed diets with higher content of lipid oxidation products, selenium, vitamin E and vitamin A. W. Poult. Sci. J. 58:357-364.