5-3-2008

Effects of individual and combinatorial electrolyte solutions comprised of various carbohydrates, salts, and stimulants injected into broiler hatching eggs on subsequent late term embryogenesis and post hatch performance through tendays of age

Brenna Mariechen McGruder

EFFECTS OF INDIVIDUAL AND COMBINATORIAL ELECTROLYTE SOLUTIONS COMPRISED OF VARIOUS CARBOHYDRATES, SALTS, AND STIMULANTS INJECTED INTO BROILER HATCHING EGGS ON SUBSEQUENT LATE TERM EMBRYOGENESIS AND POST HATCH PERFORMANCE THROUGH TENDAYS OF AGE

By

Brenna Mariechen McGruder

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agriculture
in the Department of Poultry Science

Mississippi State, Mississippi
May 2008

EFFECTS OF INDIVIDUAL AND COMBINATORIAL ELECTROLYTE SOLUTIONS COMPRISED OF VARIOUS CARBOHYDRATES, SALTS, AND STIMULANTS INJECTED INTO BROILER HATCHING EGGS ON SUBSEQUENT LATE TERM EMBRYOGENESIS AND POST HATCH PERFORMANCE THROUGH TEN DAYS OF AGE

By

Brenna Mariechen McGruder

Approved:

E. David Peebles Professor, Dept. of Poultry Science (Major Professor and Director of Thesis)	Dwaine Braasch Assistant Professor, Dept. of Biochemistry and Molecular Biology (Committee Member)
Robert Keirs Associate Professor Emeritus, Dept. of Basic Sciences, College of Veterinary Medicine (Committee Member)	John Bailey Associate Professor, Dept. of Clinical Science, College of Veterinary Medicine (Committee Member)
Michael Kidd Professor, Dept. of Poultry Science (Graduate Coordinator and Department Head of Poultry Science)	Vance H. Watson Dean of the College of Agriculture and Life Science

Name: Brenna Mariechen McGruder

Date of Degree: May 3, 2008

Institution: Mississippi State University

Major Field: Poultry Science

Major Professor: Dr. E. David Peebles

Title of study: EFFECTS OF INDIVIDUAL AND COMBINATORIAL ELECTROLYTE SOLUTIONS COMPRISED OF VARIOUS CARBOHYDRATES, SALTS, AND STIMULANTS INJECTED INTO BROILER HATCHING EGGS ON SUBSEQUENT LATE TERM EMBRYOGENESIS AND POST HATCH PERFORMANCE THROUGH TEN DAYS OF AGE.

Pages in Study: 118

Candidate for degree of Master of Science

In this study, the effects of individual and combinatorial electrolyte solution comprised of carbohydrate, salt, and a stimulant injected into broiler hatching eggs on subsequent late term embryogenesis and post hatch performance up to D 10 were evaluated in separate trials. The effects of solution concentration and volume on embryo body weight and yolk reserves were likewise considered, and limited posthatch growth, body weight, and yolk sac effects of a compound electrolyte solution were evaluated. The individual and combinatorial effects of injected solutions were examined in an effort to yield the maximum physiological growth effects on embryogenesis from the metabolic pathways in which these compounds are involved. Limited effects on embryo and post

hatch mortality were found. However, both post hatch yolk reserves and liver weights were affected by *in ovo* injection. These effects could be key in increasing bird weight by processing day.

DEDICATION

I would like to dedicate this thesis to my parents, Merle and Mariechen McGruder. Dad, I'm still trying to figure out which came first, the chicken or the egg.

ACKNOWLEDGEMENTS

First I must acknowledge the efforts of Dr. David Peebles. His willingness to introduce me to the world of poultry science with such patience was a reflection on how much he enjoys the field. His faith in me as a student and as a scientist is something I will always remember. I also must acknowledge Rafael Correa and Dr. Mark Dekich, and all the people at Avitech LLC, without their donation and support this research would not have been possible. I must also acknowledge, with a whole heart, Dr. John Boyle, who impacted my life from the first day I set foot on the campus at Mississippi State. His belief in me is what drives me to be a better scientist and a better person, and I only hope to one day live up to his example. Ms. Sharon Whitmarsh went above and beyond her call to duty as an excellent technician, computer master, and a wonderful lab mom. I would also like to acknowledge a man who knows everything and was more than willing to share it all with me, Dr. Robert Keirs. I would like to praise Dr. Braasch for all his help, he is a man of infinite amount of knowledge and wisdom and was always more than willing to answer questions I only thought I knew the answer to. To Dr. Bailey I extend my sincerest thanks, your kind words and wonderful ideas helped me find clarity. I would also like to thank the faculty and staff in the Department of Poultry Science. I

must thank Dr. Holly Parker, who helped me with everything from lab work to school work, and who was always there to remind me, “This too shall pass.” Also to Dr. Kidd who was always there with a story about how when he was in graduate school, and although they never made sense they did distract me for a few minutes, and that was always worth something. All of you were patient as I slowly began to understand, you were eager to answer any questions, to share your knowledge with me, and you helped me learn more about myself. I would like to extend my gratitude to Kristin Viscione, an excellent peer mentor, friend, and partner in crime, you were a huge help. And to my friends, especially Anna Oberhoffer and Christine Schudrowitz, who always were there to support me and to help me work through my ideas.

I must acknowledge the efforts of my family, who were more than willing to listen to me rant and ramble despite having no idea what I was talking about. I know you tried to understand. To my mom and dad, Mariechen and Merle McGruder, who supported me in every way parents can and were tolerant, to an extent, of the fact that I never called home. They are the reason for my efforts and I can only hope I have made them proud. To my little sister, Brittany, you were always there to make me laugh and to go out to lunch with me, as long as I paid. To all of my family, I can only say thank you.

Omnia mea mecum porto. Homo doctus in se divitias semper habet.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURES Page

CHAPTER I

INTRODUCTION

Over the years the poultry industry has practiced selective breeding to achieve the hardy and productive lines of birds that we have today. However, as we approach a new generation of genomics and proteomics, it is of interest to know if the genetic potential in the current commercial lines of poultry is fully realized. There is much potential in attempting to discover if a higher level of performance may be achieved if birds are provided optimum nutrition and husbandry. In this current research, materials and methods are introduced through automated *in ovo* injection in an effort to evaluate effects on mortality, body weight gains, and yolk sac reserves of Day 18 embryos and post hatch chicks up to 10 d. The materials injected at Days 16 or 18 of incubation include various salts, carbohydrates and stimulants along with the current vaccine regimen. By considering nutrient metabolic efficiency and energy output, various biochemical pathways and pathway entry points are utilized to optimize broiler growout performance.

The objective of this study was to explore the use of various metabolic intermediate solutions, via their proposed mechanisms in cellular cycles and the responses they invoke *in vivo.* The relative effectiveness of various salts, carbohydrates, stimulants, and high energy compounds individually and in combination were determined. The embryonic evaluation of these solutions, individually and in

combination, may allow for their optimal formulation for maximum effectiveness. It is hypothesized that the electrolyte solutions will have an effect on embryos and post hatch chicks, positive effects as monitored by body weight, yolk sac reserves, mortality and post hatch growth. It is also suggested that the injection of one of several stimulants may have a specific effect on the pipping response of embryos and may thereby increase hatchability of. Of the parameters which may be affected include decrease late mortality, decrease length of incubation, and narrow the window of hatch time.

CHAPTER II

REVIEW OF LITERATURE

Over the years the poultry industry has worked to select for economic performance in growth, feed efficiency, and breast muscle yield. As a new generation of genomics and proteomics approaches one must question whether growth, meat yield, and livability options have been maximized through the current genetic breeding. While the genetics continue to be optimized, the care and management needs of the various developed poultry lines have not undergone as extensive of a review. Physiological stimulation through better feeding and the emergence of newer *in ovo* technology may offer potential for evaluating the contribution of physiology on performance. *In ovo* injection, a relatively recent innovation, represents a labor saving enhancement for the vaccination of poultry against very costly diseases (Johnston *et al.,* 1997). Since its inception, most of the research in automated *in ovo* injection has included the optimizing of mechanical elements or parameters like needle type, needle length, injection site and deposition of vaccines relative to the embryo. To date, efforts employing *in ovo* technology have not focused on the potential for advancing avian physiology and development. Previous studies have shown that *in ovo* injections of feeding solutions are capable of maximizing broiler efficiency through breast meat yield (Uni *et al.*, 2005). It

is possible that these injections can be adapted to work synergistically with the current vaccine regimen.

In ovo Technologies

The current use of *in ovo* automated mass injection allows mass vaccination in a manner that is both safe to the workers and the broiler embryos. This methodology is also cost effective (Williams, 2005). Currently the poultry industry and researchers are looking for methods to improve current *in ovo* technologies and to broaden its' uses in poultry management.

Uses of Injections In ovo

Automated mass vaccination is currently the main use for *in ovo* technologies in the poultry industry. The injection of vaccine into eggs during transfer results in a cost efficient use of manpower while increasing the number of properly vaccinated animals (Williams, 2005; Johnston *et al.*, 1997). To date the main vaccine delivered in this manner is the Marek's disease vaccine. Marek's disease is a herpes-like lymphoproliterative virus that cost the industry $1 billion dollars worldwide in 2000 (Tulman *et al.*, 2000). *In ovo* injection suggests a delivery that is faster, more uniform, and less stressful on the animal (Williams *et al.*, 2005). This type of vaccination is associated with decreases mortality in the first 2 weeks of post hatch growth when compared to previous injection methods (Johnston, 1997).

Recent studies have begun to utilize this technology for more than just vaccination applications. Studies utilizing the injection of carbohydrates (Uni *et al.*, 2005; Foye *et al.*, 2006), metabolic intermediates (Uni *et al.*, 2005; Foye *et al.*, 2006), proteins (Uni *et al.*, 2005; Foye *et al.*, 2006), and amino acids (Zhai, *et al.*, 2008; Foye *et al.*, 2006; Ohata *et al.*, 2001) have been preformed. Uni *et al.* (2005) and Foye *et al.* (2006) investigated the use of *in ovo* feeding and its' application to broilers and turkeys.

In ovo Injections of Metabolic Solutions

U.S. Patent 6,592,878 B2 states that the *in ovo* feeding is "accomplished by administering the nutrient composition and/or enteric modulator into the amnion where it is then orally ingested by the subject". This claim implies that the only manner through which feeding solutions enter the embryo's body is when the amnionic fluid is ingested by the embryo prior to hatch (Uni *et al.*, 2003). This scenario assumes a static situation, meaning that the amniotic fluid remains where it is until it begins to be actively ingested by the embryo. This may not be congruent with research that describes the situation as more dynamic, whereby there is active fluid exchange throughout the organism (Jochemsen and Jeurissen, 2002; Rol'nik, 1970; Ragonzia, 1957). Other *in ovo* research concluded that substances can be taken into the embryo from the amnionic fluid, and can be located in various organs prior to hatch (Jochemsen and Jeurissen, 2002). Several types of solutions were used to track the amniotic fluid uptake by the embryo (soluble, insoluble and viral solutions). The uptake of the injected materials is more expedient and

dispersive in Day 18 embryos than Day 16. Day 16 embryos required 48 hours for all embryos tested to show evidence of the injectable, although approximately half showed positive uptake in the observed organs at 24 hours. Whereas embryos injected at Day 18 showed the injectable in the tested organs 24 hours post injection. For all solutions tested it was determined that all solutions types were easily assimilated. However, it has not been conclusively proven that amniotic fluid is actively or passively ingested (Uni *et al.*, 2005; Uni, *et al.*, 2003; Jochemsen and Jeurissen, 2002; Ragozina, 1957).

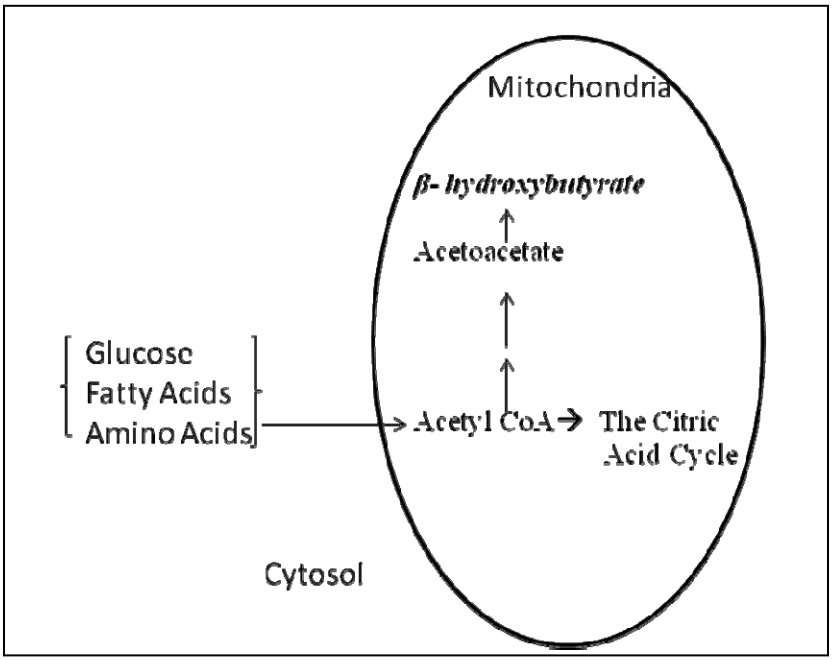

Figure 2.1. Illustration of β-hydroxybutyrate use in the cell (Devlin, 1997).

β-hydroxybutyrate was used as a leucine metabolite that was found to decrease adult chicken mortality, increase carcass yield, and prevent excessive muscle proteolysis during metabolic stress by previous investigators (Uni *et al.,* 2005). β-hydroxybutyrate

is a product of acetoacetate metabolism during ketogeneisis (Figure 1.1). This compound has been extensively tested in Day 18 injections of embryos, and was found to increase hatch weight and carcass yield (Foye *et al.*, 2006; Uni *et al.*, 2005; Tako *et al.*, 2004). Ohtsu *et al.* (2003) reported that the natural concentration of β-hydroxybutyrate was most concentrated in the blood at hatch, after which there was a marked decrease from hatch to 3 days post hatch. Muscle to blood ratio of the β-hydroxybutyrate concentrations immediate to hatch (Day 20 to hatch) were significantly higher than concentrations in the post hatch chick. The uptake of β-hydroxybutyrate by muscles is substantial. Ketogenesis, if it occurs, would occurs in muscles immediately before and after the hatching process. β-hydroxybutyrate is reported to have a greater response when injected alone than when injected with egg white protein.

However, egg white protein was shown to have a better response than β-hydroxybutyrate when injected into eggs at Day 18 (Foye *et al.*, 2006). Egg white contains ovalbumin, ovotransferrin, ovomucoid, globulins, lysozyme, ovomucin, and avidin (Romanoff, 1960). These proteins could serve various nutritive functions and supplement the endogenous protein content of the egg. Egg white protein conferred a better breast growth response than the control, β-hydroxybutyrate (individual), or the β-hydroxybutyrate + egg white protein injection solutions. Body weight increases due to the injection of these solutions leveled off at Day 3 posthatch. By Day 7 posthatch all treatment groups had outgrown the control (Uni *et al.,* 2005). The increase that was noticed at hatch is attributed to a larger breast muscle, the only muscle independently

weighed. The addition of egg white protein is theorized to conserve any muscle damage incurred during hatching either by allowing for a buildup of muscle prior to hatch or by having more products available for muscle regeneration.

In ovo Development

General Development

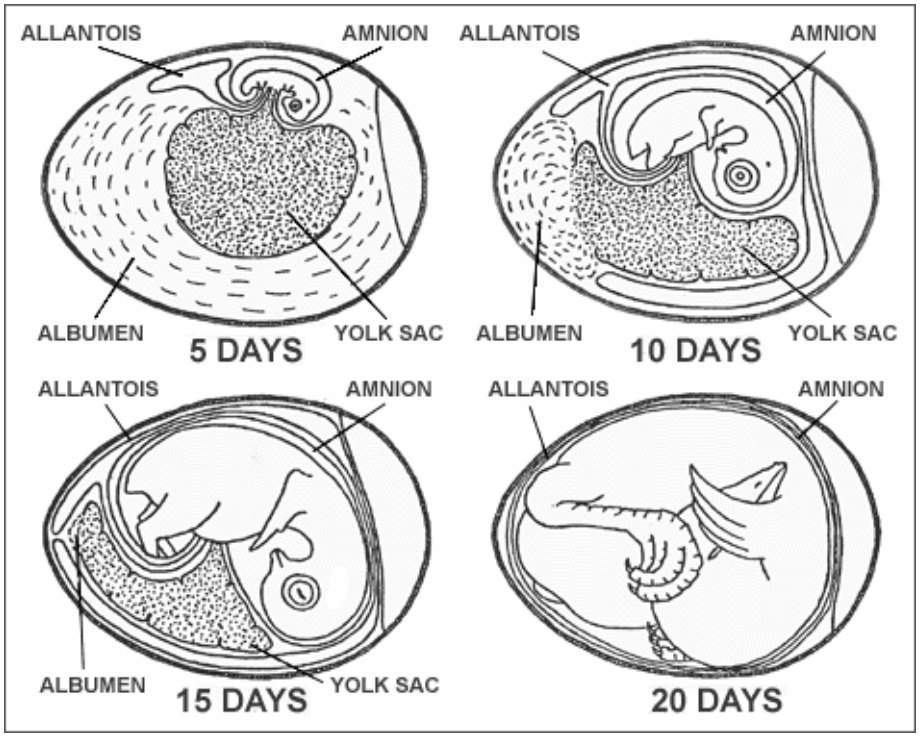

Figure 2.2. Diagram of the development of the avian embryo and select extraembryonic membranes. (Parkhurst, 1988)

The development of the avian embryo is mainly considered as three major stages: early, mid, and late. The early developmental period is the time of major organ development

and some external feature development. It is hallmarked by the formation of the heart on Day 2 and the complete development of the vascular system by Day 4 (Table 1.1; Romanoff and Romanoff, 1960). Mid- developmental periods are characterized by rapid body movement and a more "bird-like" appearance of the embryo. During this period the digestive tract is fully formed and the small intestines are external to the embryo body proper. During late developmental period the small intestines are drawn into the abdomen, on Day 15, and the remainders of the extra embryonic organs (yolk sac and amnionic fluid; Fig. 2.2) have begun to be taken into the body in preparation for hatch. By Day 19 of development, all the organs should be internal and most of the extraembryonic membranes should be gone. On Day 19 an internal pip of the inner membrane should occur, marking the first time the chick breathes oxygen. Day 20 will show outer eggshell pipping. Hatching, in the domestic chicken, occurs between late in Day 20 to 21 (Rol'nik, 1970; Romanoff and Romanoff, 1960).

Development of adult avian organs (i.e. lungs and digestive tract) is considered slow in comparison to body and extraembryonic membranes (Rol'nik, 1970). Therefore, for the avian embryo to survive and develop without the use of the adult organs the embryos must utilize the function of temporary organs, also known as the extraembryonic membranes. These membranes perform a critical function for the embryo, but would serve no purpose post hatch and so these membranes atrophy or are ingested before hatch. The formation of these membranes is semiautonomous in the avian embryo (Everaet *et al.*, 2006). Development of these membranes continued independently for a

short time after the embryo was killed. If membrane synthesis was inhibited chemically (by amiloride) the embryo still continued to develop for a short time, but then died. These external organs consist of the yolk sac, amnion/ chorion, and the allantois.

Yolk Sac

The main function of the yolk sac is the absorption of nutrients from the yolk and transfer of these nutrients to the embryo (Romanoff and Romanoff, 1967; Rol'nik, 1970). The yolk sac is one of the earliest formed membranes (Rol'nick, 1970). The yolk has a pH of 6.0 at the time of lay, and this pH increases slightly (6.5) during development (Stern, 1991). During 2 to 3 days of incubation the yolk sac distinguishes itself from the embryo, occurring simultaneous to the formation of the head, caudal, and lattice lining body folds. Also, during this time, the vitelline arteries and veins connect to form the extraembryonic circulation (Table 1.1). Growth of the yolk sac proceeds up to Day 11, at which time there is complete closure of the yolk sac (Rol'nik, 1970). During development, uptake of the yolk is the major source of nutrition for the embryo (Romanoff, 1960). During incubation the yolk sac absorbs three times its own weight in food material (Romanoff and Romanoff, 1967). It is important to note that while in the egg, the embryo has a major dependency on lipids, via the yolk, for metabolic purposes. Based on 100 g of yolk tested the yolk contains 86 g phospholipids, 4 g triacylglycerols and 10 g cholesterol (Nielson, 2003). Palmaitic (16:0) and oleic (18:1n-9) are the major

Table 2.1. Developmental highlights of avian development (*Gallus*)

	Time of Development		
	D Early (1-7 d)	D Mid (8-14 d)	D Late (15-21 d)
Embryo	D 1 Primitive streak form[1] D 2 Heart forms[1] D 6 Voluntary movement, beak forms[1]	D 8 Feather genus appear in tracts[1] D 12 Down and scales appear[1] D 14 Head moves to large end[1]	D 17 Beak under right wing, toward lower air sac D 19 Internal pip, pulmonary respiration begins[1] D 20 External pip[1]
Internal Organs	D 1 Foregut forms[1] D 2 Heart, brain, eyes, and blood vessels form D 4 All organs present[1]	D 11 Abdominal wall and intestines apparent[1]	D 15 Small intestines taken into abdomen[1]
Yolk Sac	D 1 Formation[1] D2 Vitelline arteries arise and connect extraembryonic circulation[2]	D 11 Complete closure of sac[1]	D 19 Remaining yolk taken into the abdomen[1]
Amnion/Chorion	D 3 Amnion forms and closes[2] D 4 Begin to swallow amniotic fluid[3] D 5 Contractions begin[2]	D 13 Albumen influxes in[2] D 14 Chorioamnionic duct formed, embryo actively swallows fluid [2]	D 19 All fluid swallowed[2]
Allantois	D3 Appears[1] D 5 Allantoic wall fuses with the chorionic mesodermal[1] D 6 Allantoic cavity fills with fluid	D10 Maximum weight[1] D 11 Lines entire shell membrane[1]	D 15 Reaches maximum weight[1,2] D 19 Embryo pips and pulmonary respiration begins[1]
Reference Key: [1](Romanoff, 1960), [2](Rol'nik, 1970), [3](Ragozina, 1957)			

acyl components, and the major poly-unsaturated fatty acid component is linoleic acid (18:2n-6) (Speake, *et al.*, 1998; Reidy, *et al.*, 1998). It was found that by Day 5 post hatch, approximately 90% of all lipids associated with the yolk complex had been absorbed by the chick (Noble *et al.*, 1989). "During the last few days of the developmental period, the loss of solids by the yolk is very rapid. This can be accounted for by the structural formation of the embryo, and the fats of the yolk are being utilized for the rapidly increasing expenditure of energy" (Romanoff and Romanoff, 1967). The yolk is found to decrease slightly at the midpoint of embryonic development, and then is somewhat replenished (Romanoff and Romanoff, 1967). It is unknown where this replenishing source comes from. Only a vestigial amount of yolk is found at 5 to 6 days post hatch. In the newly hatched chick there was approximately 8 grams of residual yolk matter containing 1.7 grams of lipid (28%) that remained to be absorbed (Noble *et al.*, 1989). Post hatch growth of the liver was associated with the fat that was present in the embryonic chick reserves, and the changes noted in the lipid and fatty acid composition of the liver were indicative of the rapid alteration of the role of the liver in lipid metabolism of the new chick (Nobel *et al.*, 1998).

Amnion and Chorion

The amnion is believed to provide mechanical protection, fluid balance for the external pressure, and it enables the embryo to change shape and spatial position in the egg without hazard to the newly developing organs (Rol'nik, 1970). The fluid is of a

"unique ionic composition" and has a high osmotic pressure which is believed to contribute to the fluid movement in the egg in the later stages of incubation (Ar, 1991). The amnion and chorion extraembryonic membranes are formed from the extraembryonic somatopleure from hour 30 of development (Rol'nik, 1970). The extraembryonic somatopleure forms the head fold of the chorioamniotic layers after which the lateral chorioamniotic fold and a caudal fold appears. The lateral chorioamniotic folds flow towards each other, while the caudal fold grows toward the head. When all folds meet, the portions of the membrane fuse over the embryo and form a sac (Table 1.1). The outer walls of the folds move away from the inner walls and form the chorion. The internal walls form the amnion (Figure 1.2). As the chorion grows it forms a fold and adheres to the wall of the amnion resulting in a chorio-amniotic fusion. The chorioamniotic fusion is disrupted on Day 12 and forms a chorioamniotic duct. The amnionic fluid reaches its maximum volume on Day 13, where its weight is approximately 8-9% of the eggs initial weight. After Day13 the amount of amnionic fluid increases due to the influx of albumen, and this albumen and amnionic fluid mixture is what the chick is imbibing during the latter half of incubation (Ar, 1991; Rol'nik, 1970).

Ion movement plays an important role in early embryo cell differentiation and in supporting the water movement later in incubation (Ar, 1991; Stern, 1991; Romanoff and Romanoff, 1967). The amniotic fluids contain mainly chlorides (Romanoff and Romanoff, 1967; Rol'nik, 1970). Romanoff and Romanoff (1967) noted that between the

ages of 11 and 15 days of incubation the ion content in the extraembryonic membranes shift in a predictable manner. As embryonic age increases, sodium ions decrease while potassium ions increase in the amnion (Romanoff and Romanoff, 1967).

Another critical function of the amnion is its contractions. These contractions are believed to improve metabolic reactions and to bathe the embryo by keeping fluids moving (Rol'nik, 1970). The amnion folds close at Day 3. At Day 5, due to fluid, the amnion increases in size and begins to contract. At 3 to 4 days of incubation, separate muscle fibers are visible and these fibers form a common muscle syncytium during 5 to 7 days of incubation. The muscular structure of the amnion develops in order to form a contractile network. Then as the embryo becomes older and begins to fill the amnionic space the once highly developed membrane becomes greatly simplified. It is after this degeneration of the contractile network that the injection would be given. By this time the contractions would have ceased and the membrane would be simplified in preparation of hatch.

Swallowing plays an important role in the embryo's development and on the proposed success of *in ovo* vaccination. The fluid that the embryo would have access to swallow is the fluid immediately surrounding it, amnionic fluid. On Day 9 of development, the chick begins to swallow amnionic fluid due to the amnion contractions. Ragozina (1961) discusses swallowing as a transition to an intraintestinal form of feeding, as well as compensation for fluid deficiency when the embryo is no longer getting sufficient fluid from the yolk. This swallowing may not have a detectible effect

on overall volume of the amnionic fluid. Since there are several compartments pumping fluid into the amnion it is difficult to tell how much swallowing would decrease the total fluid volume. On Day 14, the embryo begins to actively swallow amniotic fluid, which is now mixed with albumen, in preparation for hatch. As a result of this albumen consumption, protein metabolism of the embryo is activated and this results in increased weight and plumage formation (Romanoff and Romanoff, 1967). There is no albumen left by Day 19. This is important because in some cases amnionic vaccinations are given at Day 19, which would make the site access difficult.

Allantois

The allantois is perceived to function primarily as a respiratory organ. The allantois oxygenates the blood of the embryo and relieves the carbon dioxide (Patten, 1951). It is the only organ of gas exchange from the end of Day 8 until Day 19, when internal pipping would initiate pulmonary respiration. The allantois is a highly vascularized membrane (Table 1.1; Rol'nik, 1970). Little is known about the allantois other than it must contain fluid (Ar, 1991). The allantoic membrane appears as a distinct structure on Day 3 of development, fills with fluid, and reaches its maximum wet weight at Day 10 (Romanoff and Romanoff, 1960; Rol'nik, 1970; Patten, 1951). During this time the fluid pH drops from 8.0 (at time of formation) to 5.0 to 6.0 (shortly before hatch) (Rol'nik, 1970). The allantois arises directly from the embryonic tissues as an excrescence of the posterior wall of the hindgut, moving the chorion aside from the

amnion and yolk sac. The allantois then presses tightly to the shell near the small end of the egg (Rol'nik 1970). By Day 11, the allantois grows to such an extent that it lines the entire shell membrane, and by Day 15 it reaches it maximum weight and there is almost no albumen left in the albumen sac because it has all moved into the allantoic membrane. A second rise in weight occurs near hatching time due to fluid shifts from other areas, such as the albumen sac. (Romanoff and Romanoff, 1967). The volume of the allantoic fluid seems to reflect the embryo's balance of urinary input and reabsorption via the chorioallantois (Ar, 1991). It was found that when water was available then the membrane stored a hypo-osmotic solution. The water present in the membrane may be recycled in dehydrated eggs to prevent large moisture shifts in the embryo (Hoyte, 1979). The total nitrogen content is found to increase with the growth of the membrane, with a fivefold increase in nitrogen between 8 to 21 d (Romanoff and Romanoff, 1967). An inverse relation of sodium and potassium concentration is present in the allantois between 11 and 15 days of incubation (Romanoff and Romanoff, 1976). Sodium decreases while the potassium content increases.

Water in the Egg

In ovo Water Movements

During incubation avian eggs are never known to gain water from the environment (Ar, 1991). In terms of water loss fertile eggs do not differ from non fertile eggs for the first half of incubation, illustrating how partial pressure of water vapor inside

the egg plays a role in water loss (Ar, 1991; Romanoff and Romanoff, 1967; Christensen and McCorkle, 1982). The main source of water for the newly developing embryo is found in the albumen (85-95%), with the yolk being less (42-65%) (Ar, 1991). This water is both used for metabolic purposes and is lost as vapor to the atmosphere during incubation. Many of the water shifts in the egg are believed to be functions of osmotic pressure.

The albumen is found to pump water into the yolk during the first 20% of incubation in both fertile and, to a lesser extent, non-fertile eggs (Ar, 1991). This is thought to occur due to effects of the initial osmotic pressure differences between the two compartments. This decrease is affected somewhat by continuous evaporation of water from the albumen. The decrease in fluid is mainly thought to be caused by the ion pumping action of the blastoderm disc into a space under the newly developing embryo, which forms a sub-embryonic fluid compartment (New, 1956).

All extraembryonic membranes are reported to reach final size and position approximately halfway through incubation (Ar, 1991; Romanoff and Romanoff, 1967; Rol'nik, 1970). After this midway point all embryonic uptake of water from the different compartments is believed to be osmotically driven (Ar, 1991). Embryo moisture has been found to decrease sigmoidally between 6 to 21 days. However, this moisture decrease may be offset by the increase in embryo fat between 12 to 21 days (Peebles, *et al.*, 1999). It is believed that the allantois serves to store and recycle fluid in order to prevent any large changes in embryo water content (Hoyt, 1979). Throughout

incubation it is believed that while fluid reserves decrease throughout the all compartments, the embryo will maintain its' own moisture concentration (Tullet and Burton, 1982). The total water concentration of the fresh egg has been shown to equal that of the newly hatched chick (Ar and Rahn, 1980).

Electrolytes

A solution containing free ions that behaves as an electrically conductive medium is known as an ionic solution, or an electrolyte. These solutions possess many physical, biological, and medical functions. In the simplest form an electrolyte is water containing a single salt. However, usually these solutions are more complex and contain many salts, sugars (i.e. glucose or sucrose), and minerals (Rehrer, 2001; Lima *et al.*, 2002; Snyder, 1991). These electrolytes, if added, may create an effect on water movement in the egg during incubation.

Effects of Potassium and Sodium

Most animal cells strive to maintain low intracellular sodium and high intracellular potassium for function (Voet and Voet, 2004; Devlin, 1997; Kernan, 1980). For example, the concentration of sodium is 10 mM intracellularly and 100 to 140 mM extracellularly. Conversely, the cell tries to maintain 100 to 140 mM potassium intracellularly and 5 to 10 mM extracellularly. It is the high intracellular potassium concentration that helps create and maintain the intracellular gradient charge and allow

for the creation of membrane potential (Voet and Voet, 2004; Devlin, 1997; Kernan, 1980). These ions aid in the maintenance of cell volume, cell excitability, active transport functions, and passive transport regulation. It was found that injected potassium is rapidly lost from the blood (Kernan, 1980). Potassium becomes most concentrated in the renal tissue, and approximately 20% is lost in the urine. Lungs and intestines have a high potassium turnover, whereas muscles have a comparatively lower turnover (Kernan, 1980). In a comparative study it was found that electrolyte solutions containing potassium, sodium, or sugar all raised plasma volumes after 2 hours of rehydration (Neilsen *et al.,* 1986). These studies reported that the sodium drink produce a primary effect in filling the extracellular compartment, where the potassium and sugar drinks increased intracellular compartment volumes.

Effects of Carbohydrates and Minerals

Glucose is a known electrolyte balancer, functioning by increasing water absorption (Rehrer, 2001; Snyder, 1991). Also, some electrolyte solutions evaluate the use of glucolytic amino acids for use in the place of glucose (Zhai *et al.*, 2008; Lima *et al.*, 2002). Carbohydrate ingestion during prolonged exercise (like hatching) can aid performance indirectly through increased glucose oxidation but also, importantly, through enhanced water absorption. It has been found that carbohydrate inclusion in a solution affects the net rate of water assimilation, and is important to supplement endogenous reserves as a substrate for exercising muscles (Rehrer, 2001). Glucose and/or

glucose-containing carbohydrates (e.g. sucrose, maltose) at concentrations of 3 to 5% weight/volume are added to electrolyte solutions to enhance water absorption. Carbohydrate concentrations above this may be advantageous in terms of glucose oxidation and maintaining exercise intensity. However, if these carbohydrate concentrations are too high there will be a reduced net rate of water absorption.

In search of a solution that would be beneficial in terms of nutrition and hydration the potential of the World Health Organization's (WHO) oral rehydration solution has been studied (Pulungsih *et al.*, 2006; Islam *et al.*, 2004). WHO promotes two forms of oral rehydration solution: the standard Oral Rehydration Solution (ORS) and the newer formula designed for children (ReSoMal). Both solutions include complex (sucrose) and simple sugars (glucose), as well as physiological salts and minerals (Pulungsih *et al.*, 2006). However, ORS was reformulated to decrease the concentrations of sodium, glucose, and chloride, all of which are dangerous if concentrations are too high. Excess concentrations of these molecules may exacerbate an electrolyte imbalance and induce an excess loss of water (Islam *et al.,* 2004; Rehrer, 2001). In the new reformulation the ReSoMal maintains the original ORS concentration of potassium and citrate and decreses sodium and glucose concentrations. Islam *et al.* (2004) reported that in dehydrated, but well-nourished animals, the administration of ReSoMal allowed for less sodium and more potassium to be absorbed. This reformulation brought about intestinal water absorption, which allowed the kidney and large intestine to better salvage water and electrolytes. ReSoMal also contains copper.

Copper was found to result in a positive effect on intestinal physiology of broilers, causing a decrease in villi blunting as compared to subtheraputic antibiotic treatment (Arias, *et al.*, 2006). An increase in weight at the time of processing was also noticed in the broilers that were supplemented exogenous copper in the feed (Arias, *et al.*, 2006). The addition of copper may stimulate growth and general health of broilers if added to the diet via the comparative lengthening of the villi. This villi lengthening would lead to increased absorbance during digestion. Although it is unknown if there would be an effect in embryos or chicks, there is the potential for a positive outcome.

Effects of Stimulants

Stimulants may prove to be important in the poultry industry because of the potential to stimulate embryos in hatching. In one study there was found 2.0 and 2.8% mortality in embryos at Day 20 and 21, respectively (Kuurman *et al.*, 2001). It is unknown why these embryos failed to hatch. It may be assumed that some embryos fail to hatch because of poor stimulation of the muscles involved in pipping, although there was no literature found on the topic. This is where the addition of a stimulant may show an effect.

Caffeine is a well known and well studied stimulant. It is known that cells respond differentially to caffeine. Where some can utilize it others require or better utilize one of the metabolic derivatives (Fredholm *et al.*, 1999; Siegal, 1999). Xanthenes

(i.e. caffeine and theophylline) are known to inhibit the activity of phosphodiesterase, thereby increasing the half-life of cyclic adenosine monophosphate (cAMP) in the cell

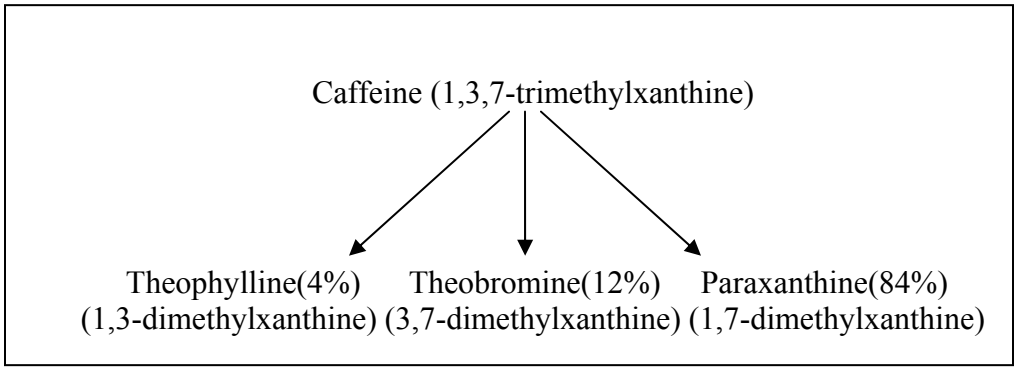

Figure 2.3. Breakdown of Caffeine (Fredholm *et al.*, 1999)

(Siegal, 1999; Fredholm *et al.*, 1999). This increase of cAMP activity further serves to potentiate agents that act by stimulating adenylyl cyclase. It acts in this manner because the organic structures of xanthenes are similar to that of adenosine. Theophylline is a product of the breakdown of caffeine, and both are xanthenes (Fredholm *et al.*, 1999; Siegal, 1999). Theophylline is a well known drug that causes relaxation of the bronchii and is used in patients with bronchitis (Devlin, 1997). It is possible that either of the products of caffeine or theophylline could prove beneficial to the avian species, especially in regards to the pipping muscles. However, increased intracellular cAMP in rapidly proliferating cells like those that would be common to a developing embryo is associated with an inhibition of mitosis (Yokoyama *et al.*, 1983). Yokoyama *et al.* (1983) examined the responses of embryonic chicks given 21 mM theophylline. At the

concentration of 21 mM aortic aneurysms were induced in these embryos at Day 5 of development. It is unknown if embryos at later stages of development would be more tolerant to similar or higher concentrations of theophylline. Most of the rapid cellular division would be complete by the late stages of development and the embryo would be focusing energies on general growth rather than rapid proliferation and cell differentiation.

Phosphocreatine and creatine work together to store energy in vertebrate cells (Voet and Voet, 2004; Devlin, 1997; Mathews, 1990). Phosphagens, like phosphocreatine allow for an ATP "buffer" in cells that contain creatine kinase, and allow for the storage of the high energy compound (Voet and Voet, 2004). Creatine and phosphocreatine are synthesized by reversible phosphorylation where ATP phosphorylates creatine yielding phosphocreatine and ADP (creatine + ATP ↔ phosphocreatine + ADP) (Voet and Voet, 2004; Devlin, 1997). Because this reaction occurs while the cell is resting and ATP concentration is high, the reaction proceeds so that more phosphocreatine is present. As a result, when the cell is in a state of high activity, such as during pipping and hatching, the reaction reverses to create more creatine and ATP. As much as 70% of the immediate high-energy stores contained within the mammalian skeletal muscle are in the form of phosphocreatine (Clark, 1997). However, in natural conditions there is only enough phosphocreatine stored for the cell to be able to exert itself for a few seconds under maximum activity, or a few minutes under normal activity (Clark, 1997). Therefore, it is possible that additional sources of creatine

with added phosphate groups or phosphocreatine would allow for longer period of cellular activity.

Saks *et al.* (2000) studied the role of creatine and phosphocreatine in the regulation of mitochondrial respiration. It was shown that the addition of creatine had no effect on ADP concentration in the medium. The ADP concentration remained the same. Concentrations of ATP were not altered in the experiment, just the creatine concentration. This illustrates that creatine is just a storage molecule and, as such, has no effect on the total cellular concentration of ATP. However, it was found that the mitochondrial respiration rate was increased by three fold. It is proposed that creatine, by activating creatine kinase, can directly control mitochondrial energy production. It was hypothesized that at higher workloads creatine kinase should be upregulated by increasing creatine concentrations and decreasing phosophocreatine concentrations. It is known that the cellular concentration of creatine is determined by specific transporters and creatine transport into the cell is concurrent with sodium against the concentration gradient (Clark, 1997).

There is a potential drawback to the addition of phosphocreatine to a solution. If phosphocreatine is orally administered it is readily broken down in the intestinal tract to creatine, losing the high energy phosphagen (Clark, 1997). However, if injected intramuscularly or intravenously the phosphocreatine can last for 2 to 5 hours (Clark, 1997). It would be difficult to determine if the embryo actually would ingest the phosphocreatine injected *in ovo* through the digestive tract or if it would be non-

destructively taken up through a non-active digestive system, or possibly other organs (i.e. the lungs). Clark (1997) reported that creatine generates an anabolic effect on muscle tissues by causing increased protein synthesis. This may allow for the pre-build up of muscles so that at post hatch, the damage is negated, allowing for increased aerobic recovery by stimulating mitochondrial respiration and oxidative phosphorylation.

Conclusion

There are many options when it comes to evaluating the potential of broilers. One option is supplemental *in ovo* injections that can be administered along with the current vaccine regimen. There are many metabolic, nutritive, or other soluble molecules that would be suitable for compatibility to Marek's, or any other, vaccine regimen. These additives may serve in increasing the general nutritional health of the newly hatched chicks along with the additives' proven ability to stimulate post hatch growth, especially that of the breast muscle (Uni *et al.*, 2005).

References

Ar, A. 1991. Egg water movements during incubation. Avian Incubation. S.G. Tullett, ed. Butterworth-Heinemann Ltd., London, UK. 157-173.

Ar, A., H. Rahn. 1980. Water in the Avian Egg: Overall Budget of Incubation. American Zoologist. 20:373-384.

Arias, V.J. and E.A. Koutsos. 2006. Effects of Copper Source and Level on Intestinal Physiology and Growth of Broiler Chickens. Poultry Science. 85:999-1007.

Christensen, V.L., F.M. McCorkle. 1982. Turkey Egg Weight Loss and Embryonic Mortality During Incubation. Poultry Science. 61: 1209-1213.

Christensen, V.L., M.J. Wineland, G.M. Fasenko, W.E. Donaldson. 2001. Egg Storage Effects on Plasma Glucose and Supply and Demand Tissue Glycogen Concentrations of Broiler Embryos. Poultry Science. 80:1729-1735.

Clark, J.F. 1997. Creatine and Phosphocreatine: A Review of Their Use in Exercise and Sport. Journal of Athletic Training. 32: 45-51.

Devlin, T.M. 1997. Textbook of Biochemistry with Clinical Correlations. Wiley and Sons Printing. New York, New York. 206-208, 388-389, 688-689, 1065-1068.

Everaert, N., P.M. Coucke, F. Bamelis, B. Kemps, B. De Ketelaere, V. Bruggeman, J. De Baerdemaeker, E. Ducuypere. 2006. Semiautonomous Development of the Extraembryonic Membranes in the Chicken Embryo. Poultry Science. 85:1626-1631.

Foye. O.T., Z. Uni, P.R. Ferket. 2006. Effects of In Ovo Feeding Egg White Protein, β-Hydroxy-β-Methylbutyrate, and Carbohydrates on Glycogen Status and Nenonatal Growth of Turkeys. Poultry Science. 85:1185-1192.

Fredholm, B.B., K. Battig, J. Holmen, A. Nehlig, E.E. Zartau. 1999. Actions of Caffeine

in the Brain with Special Reference to Factors that Contribute to its Widespread Use. Pharmacological Reviews. 51:83.

Gal-Garber, O., S.J. Mabjeesh, D. Sklan, Z. Uni. 2003. Nutrient Transport in the Small Intestine: Na+, K+-ATPase Expression and Activity in the Small Intestine of the Chicken as Influenced by Dietary Sodium. Poultry Science. 82:1127-1133.

Hayes, V.E., and R.S. Hikida. 1976. Naturally-occurring degeneration in chick muscle development: ultrastructure and the M. complexus. Journal of Anatomy. 122:67-76.

Hoyte, D.F. 1979. Osmoregulation of Avian Embryos: The Allantois Functions Like a Toad's Bladder. Physiological Zoology. 52:354-362.

Huerta, M. and E. Stefani. 1981. Potassium and Caffeine Contractures in Fast and Slow Muscles of the Chicken. Journal of Physiology. 318:181-189.

Islam, S., M. Abley, N.H. Alam, F. Doussou, A.K.A. Chowdhury, J.F. Desjeux. 2004. Water and Electrolyte Salvage in an Animal Model of Dehydration and Malnutrition. Journal of Pediatric Gastroenterology and Nutrition. 38: 27-33.

Jochemsen, P. and S.H.M. Jeurissen. 2002. The Localization and Uptake of In Ovo Injected Soluble and Particulate Substances in the Chicken. Poultry Science. 81:1811-1817.

Johnston, P.A., H. Liu, T. O'Connell, P. Phelps, M. Bland, J. Tyczkowski, A. Kemper, T. Harding, A. Avakian, E. Haddad, C. Whitfill, R. Glidersleeve, C.A. Ricks. 1997. Applications in In Ovo Technology. Poultry Science. 76: 165-178.

Kernan, R.P. 1980. Cell Potassium. Wiley and Sons Printing. New York, New York. 120-174

Kuurman, W.W., B.A. Bailey, W.J. Koops, M. Grossman. 2001. Effect of Hatch on the Distribution for Failure of an Embryo to Survive Incubation. Poultry Science. 80:710-717.

Lima, G.H.P. Carvalho, A.A. Figuerido, A.R. Gifoni, A.M. Soares, E.A.T. Silva, R.L. Guerrant. 2002. Effects of an Alanyl-Glutamine-Based Oral Rehydration and Nutrition Therapy Solution on Electrolyte and Water Adsorption in a Rat Model of Secretory Diarrhea Induced by Choera Toxin. Nutrition. 18:458-462.

New, D.A.T. 1956. The Formation of Sub-Blastodermic Fluid in the Hen's Egg. Journal

of Embryology and Experimental Morphology. 4:221-227.

Nielsen, H. and V.K.S. Shukla. 2003, In situ solid phase extraction of lipids from Spray-dried Egg Yolk by Ethanol with Subsequent Removal of Triacylglycerols by Cold Temperature Crystallization. LWT-Food Science and Technology. 37:613-618.

Noble, R.C. and D. Ogunyemi. 1989. Lipid Changes in the Residual Yolk and Liver of the Chick Immediately after Hatching. Biology of the Neonate. 56:228-236.

Ohata, Y., M.T. Kidd, T. Ishibashi. 2001. Embryo Growth and Amino Acid Concentration Profiles on Broiler Breeder Eggs, Embryos, and Chicks After In Ovo Administration of Amino Acids. Poultry Science. 80:1420-1436.

Ohtsu, H., K. Sato, H. Nishida, Y. Akibia. 2003. High β-hydroxybutyrate concentration in liver and skeletal muscle of newly hatch chicks. Comparative Biochemistry and Physiology Part A. 134:625-629.

Parkhurst, C.R., G.J. Mountney, 1988. Poultry Meat and Egg Production.Van Nostrand Reinhold Company, New York. p80

Patten, B.M. 1951. Early Embryology of the Chick. The Blakiston Company. Philadelphia, Pennsylvania. 124-138.

Peebles, E. David, Lumu Li, Sherman L. Miller, Tomas Pansky, Sharon Whitmarsh, Mickey A. Latour, and Patrick D. Gerard, 1999. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poultry Science. 78:1435-1442.

Pulungsih, S.P., N.H. Punjabi, K. Rafli, A. Rifajati, S. Kumala, C.H. Simanjuntack, Yuwono, M. Lesmana, D. Subekti, Sutoto, O. Fontaine. 2006. Standard WHO-ORS Versus Reduced-osmolarity ORS in the Management of Cholera Patients. Journal of Health Popular Nutrition. 24: 107-112

Ragozina, M.N. 1957. Liberation of the Chick Embryo from the Vitelline Membrane and Chalaze of the Egg. DAN SSSR. 113:716

Rehrer, N.J. 2001, Fluid and Electrolyte Balance in Ultra-endurance Sport. Sports Medicine. 31:701-715.

Reidy, T.R., J.L. Atkinson, S. Leeson. 1998. Size and Components of Poult Yolk Sacs. Poultry Science. 77:639-643.

Rol'nik, V.V. 1970. Bird Embryology. Keter Press Binding. Jerusalem. 93-108.

Romanoff, A.L. 1960. The Avian Embryo. Wiley and Sons Printing. New York, New York.

Romanoff, A. L. and A. J. Romanoff. 1967. Biochemistry of the Avian Embryo. Wiley and Sons Printing. New York, New York. 144-257.

Saks, V.A., O. Kongas, M. Vendelin, L. Kay. 2000. Role of the creatine/phosphocreatine system in the regulation of mitochondrial respiration. Scandinavian Physiological Society. 168: 635-641.

Siegel, G.J., B.W. Agranoff, R.W. Albers, S.K. Fisher, M.D. Uhler. 1999. Basic Neurochemistry Molecular, Cellular, and Medical Aspects, 6th Edition. Lippincott, Williams and Wilkins Publisher. Chapter 17.

Snyder, J. D. 1991. Use and Misuse of Oral Therapy for Diarrhea: Comparison of US Practices with American Academy of Pediatrics Recommendations. Pediatrics. 87: 28–33.

Speake, B.K., A.M.B. Murray, R.C. Noble. 1998. Transport and Transfomrations of Yolk Lipids During Development of the Avian Embryo. Progress in Lipid Research. 37:1-32.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed. McGraw-Hill, New York, NY.

Stern, C.D.1991. The Sub-embryonic Fluid of the Egg of the Domestic Fowl and its Relationship to the Early Development of the Embry. Avian Incubation. S.G. Tullett, ed. Butterworth-Heinemann, Ltd. 81-87.

Tako, E., P.R. Ferket, Z. Uni. 2004. Effects of In Ovo Feeding of Carbohydrates and β-Hydroxy-β-Methylbutyrate on the Development of Chicken Intestine. Poultry Science. 83:2023-2028.

Tullet, S.B. and F.G. Burton. 1982. Factors Affecting the Weight and Water Status of the Chick at Hatch. British Poultry Science. 23:361-369.

Tulman, E.R. C.L. Afonso, Z. Lu, L. Zsak, D.L. Rock, G.F. Kutish. 2000. The Genome of a Very Virulent Marek's Disease Virus. Journal of Virology. 74:7980-7988.

Uni, Z., and P. Ferket. 2003. Enhancement of development of oviparous species by in ovo feeding. US Regular Patent US 6,592,878 B2. North Carolina State Univ., Raleigh and Yissum Res. Dev. Co., Hebrew Univ. Jerusalem, Israel.

Uni, Z., P.R. Ferket, E. Tako, O. Kedar. 2005. In ovo Feeding Improves Energy Status of Late-Term Chick Embryos. Poultry Science. 84: 764-770.

Uni, Z., E. Tako, O. Gal-Garber, D. Sklan. 2003. Morphological, Molecular, and Functional Changes in the Chicken Small Intestine of the Late-Term Embryo. Poultry Science. 82: 1747-1754.

Voet, D. and J.G. Voet. 2004. Biochemistry, 3rd edition. Wiley and Sons Printing. New York, New York.

Williams, C.J. 2005. In ovo vaccination and chick quality. International Hatchery Practice. 19:7- 13.

Yokoyama, H., R. Matsuoka, H.J. Bruyere, E.F. Gilbert, H. Uno. 1983. Light- and Electron-Microscopic Observations of Theophylline-Induced Aortic Aneurysms in Embryonic Chicks. American Journal of Pathology. 112: 258-266.

Zhai, W., S. Neuman, M.A. Latour, P.Y. Hester. 2008. The Effect of In Ovo Injection of L-Carnitine on Hatchability of White Leghorns. Poultry Science. 87:569-572.

CHAPTER III

EFFECTS OF INDIVIDUAL ELECTROLYTE SALTS, ELECTROLYTE WITH CARBOHYDRATE, AND STIMULANT SOLUTIONS ON EMBRYOGENESIS IN BROILER HATCHING EGGS

Abstract

Effects of the automated *in ovo* injection of various concentrations and volumes of a variety of physiological salts, a carbohydrate solution, and various stimulants on the livability and growth of broiler embryos were investigated. Four trials were conducted separately. Solutions were injected into the amnion of embryos at Day 16 of incubation. Embryo mortality; relative embryo weight and moisture content; relative dry embryo weight; and relative yolk sac weight and yolk moisture content were evaluated on Day 18 of incubation in each trial. Trial 1 was conducted to determine the effects of 4 salt solutions (sodium acetate, potassium chloride, and sodium dihydrogen phosphate) at various concentrations. Trial 2 was conducted to determine the effects of 2 metabolic solutions (tripotassium citrate and a carbohydrate/electrolyte solution) and a stimulant (caffeine) at various concentrations. Trial 3 was conducted to evaluate the effects of potassium chloride (5.5 mM) and sodium chloride (117 mM) at 200, 400, 800, and 1200 µL volumes. Trial 4 was conducted to compare the effects of caffeine, theophylline,

creatine, and phosphocreatine, all at a concentration of 1 mM. None of the experimental solutions used in any of the 4 trials had a significant effect on embryo mortality. In trial 1, potassium chloride, at all concentrations, increased relative yolk sac weight in comparison to non-injected controls. In trial 2, all 3 solutions, and all their concentrations, had no effect of any kind on the parameters examined. In trial 3, the 1000 and 2000 µL potassium chloride and saline volumes reduced percent incubational egg weight loss in comparison to controls. Furthermore, the 800 µL volume of saline reduced relative embryo body weight in comparison to non-injected controls. In general, any volume over 800 µL reduced incubational egg weight loss. In trial 4, relative yolk sac weight was reduced by all injected materials in comparison with the non-injected control. In conclusion, the solutions tested have potential for use separately or in combination for the commercial injection of broiler hatching eggs in order to promote subsequent hatchability and post hatch growth.

Introduction

The embryo is protected by a shell, but it also has a limited nutrient supply because it is not attached to its mother by an umbilical cord. While the enclosed environment in which avian embryos grow provides the necessary reservoirs for embryogenesis the one time supply of these resources from the mother may become limiting under the pressures of commercial production. As the embryo develops it utilizes, and eventually uses, all of its available sources of nutrition. Over the past few years, some researchers have utilized *in ovo* injection in an effort to increase chick hatch

weight and bird size at the time of processing. It has been shown by Uni *et al*. (2005) and Foye *et al*. (2005) that the injection of nutritional supplements benefit post hatch growth and the weight gain of birds. The broiler embryos (Ross) examined by Uni *et al.* (2005) showed no significant gain after *in ovo* feeding on Days 19 and 20 of embryonic development.

Herein, attempts were made to demonstrate the usefulness of metabolic substrates in the development of embryos between Days 16 and 18 of incubation. In the following experiments, the possible use of individual electrolyte solution constituents and stimulants injected *in ovo* to promote growth and development of the avian embryo were explored.

In trial 1, the use of various physiological salt solutions that may serve as more beneficial substitutes for saline were investigated. Potassium chloride is a commonly known physiological salt, and has the potential to be used as, or more, effectively as saline. This potential may become important when considering the cellular pump activity in cells, in that there is always a continuous effort to expel sodium and retain potassium intracellularly (Voet and Voet, 2004). Sodium acetate has the potential to provide both sodium and acetate. More specifically, it may provide a Na^+ cation without an associated Cl^-anion, which is harmful in high concentrations. Sodium phosphate has the potential of contributing additional phosphate to the overall metabolic system, thereby increasing the percentage production of ATP and other high energy compounds.

The carbohydrate/electrolyte nutrient solution tested in trial 2 was the only complex solution that was tested. This solution was taken from the World Health

Organization's ReSoMal. It was chosen because previous work has demonstrated the effectiveness of simple carbohydrate solutions in avian embryos (Uni *et al.*, 2005). Also, these solutions have been originally used for catch-up feeding of malnourished children, and they have shown effectiveness in maintaining hydration in animals that were well fed (Islam *et al.*, 2004). Tripotassium citrate has the potential of not only providing additional potassium for various cellular functions, but also provides the useful citrate anion as opposed to the harmful chloride ion. It may also serve as an acidity regulator and an antioxidant.

In trial 3, the comparative effects of various volumes of potassium chloride and saline were investigated. It was also utilized in order to establish any potential volume disparities, as throughout *in ovo* research the volume of trial solution has varied without cause (Uni *et al.*, 2005; Foye *et al.*, 2005; Ohata *et al.*, 2001). This trial was important to determine if the effects of potassium chloride were similar or even better, than those of saline. It also allowed for an evaluation of the effects of varying solution volume on embryogenesis. Volume as well as concentration effects must be included in order to best determine the supportive roles of the various salt and metabolic solutions on embryogenesis and posthatch growth.

In trial 4, the effects of 2 stimulants were investigated. Caffeine serves as both a metabolic stimulator and a central nervous system stimulator (Siegel *et al.*, 1999). It is proposed that the *in ovo* injection of caffeine would increase metabolic activity (which may promote growth) and would also stimulate muscle activity (which may aid in the pipping response). Theophylline was also tested because it is a metabolic product of

caffeine catabolism (Siegel *et al.*, 1999). There are certain cell types that respond better to theophylline than caffeine, and as such each form may cause a distinctive response in different cells (Devlin, 1997). To determine which would provide the best response in an avian embryo, both were tested. Also, 2 high energy compounds were examined. Creatine is a high energy storage compound found in many different cells (Voet and Voet, 2004; Devlin, 1997). However, phosphocreatine may serve as a phosphate reservoir, thereby reducing the need for ATP as a source by which to load creatine with phosphate. Phosphocreatine may be more beneficial than creatine, since it would not utilize the embryo's own nutrient and energy sources, and would subsequently reduce yolk sac absorptive requirements. The addition of phosphocreatine may allow avian muscle cells that are vital to hatching to exert themselves more during the hatching process, creating a more uniform hatch time, or even stimulating the muscles of chicks that would not have had the energy to hatch. If raw creatine is available to the cell, the individual cell could utilize this new storage capacity. It may prove beneficial to provide the additional phosphagen and allow the chick to utilize it as needed. There is the potential that more creatine, if added, would allow for better embryonic muscle stimulation and would allow for more yolk sac utilization.

Materials and Methods

Incubation

Broiler hatching eggs (Ross x Ross) were obtained from a commercial source (Peco Farms, Inc). All eggs used within a trial were taken from a common flock. Eggs used within different trials were taken from different flocks. However, all eggs were taken from flocks that were between 35 and 54 weeks of age. All eggs were held for 3 to 4 days prior to setting. Set weights were taken for individual eggs and the eggs were arranged randomly in each incubator tray level (experimental block). On each tray level, all treatments were equally represented and representative replicate groups of eggs for each treatment were set at random. The number of eggs set within each treatment replicate group for trials 1, 2, 3, and 4, were 5, 5, 10, and 10, respectively. The number of individual treatment replicate units in trials 1, 2, 3 and 4, were 4, 4, 2 and 2. In trials 1 and 2, the individual replicate units were represented by 2 tray levels in each of 2 tray columns, and in trials 3 and 4, the individual replicate units were represented by 2 tray columns on one tray level. Eggs were incubated in a Jamesway AVN single stage incubator for all trials. Incubator dry bulb temperatures were set at 37.6°C, with a variable range of 37.5 to 37.7°C during incubation. Incubator wet bulb temperatures were maintained between 26.0º and 30.0º C (i.e. 83 and 86% relative humidity).

Preparation of Solutions

All injected solutions were prepared within 2 to 5 d of injection. A 117 mM saline solution (pH 4.2) was used as a standard and there was a non-injected control (**control, ni**) in all trials. It trials 1 and 2, solutions were prepared in RhoPure water. In trial 1, all solutions were prepared to achieve a specific molarity. Solution pH was also recorded for all solutions (Table 3.1). Sodium acetate trihydrate (**NaAce**; Sigma-Aldrich) solutions were prepared at 110, 118, 125, and 130 mM. Potassium chloride (**KCl**; Fisher Scientific) solutions were prepared at molarities of 4.0, 4.5, 5.0, and 5.5 mM. Sodium phosphate monobasic (**NaH_2PO_4**; Sigma-Aldrich) solutions were prepared at molarities of 0.5, 1.0, 1.5, and 2.0 mM. In trial 2, the effectiveness of two metabolic solutions in various dilutions were evaluated. A volumetric dilution series of 1:20 (concentrate : water) for both the carbohydrate and the salt solutions was performed to accommodate lower concentrations of solutions that could not be prepared by hand. The pH was also recorded for each solution (Figure 3.2). For tripotassium citrate monohydrate (**3K Cit**; Sigma-Aldrich) the starting concentration was 0.1 M anda dilution series was used to achieve the lesser volumes (5.0 mM, 0.25 mM, and 0.25 µM concentrations, respectively). A carbohydrate/electrolyte solution (**CEN**) was diluted according to the following dilution series of full strength, 1:20, 1:40, and 1:60; with a pH range of 7.2 to 7.4. The CEN solution is that used in a recipe for catch-up feeding of malnourished children by the World Health Organization and has multiple constituents at different concentrations (Table 3.5). The final solution used in trial 2 was the stimulant caffeine (anhydrous; Sigma-Aldrich), at 1pM, 1, nM, 1µm, 1mM concentrations. The caffeine

solutions were prepared via a 1:100 volumetric dilution series. NanoPure water was used in trial 3 and the solutions were prepared in the standard method for their select molarity. In trial 3, KCl (5.5 mM) and physiological saline (117 mM) were tested at 200, 400, 800, and 1200 μL volumes. The specified concentrations for each solution were maintained at all 4 volumes tested. The pH was measured for both solutions (Figure 3.3). In trial 4, solutions were prepared in autoclaved water and were then filtered through a TRP© single use, gamma sterilized syringe filter (0.22 μm) one day prior to injection. Osmolarities and pH was recorded for all solutions (Figure 3.4). In trial 4 caffeine (anhydrous; Sigma-Aldrich), theophylline (anhydrous; Sigma-Aldrich), creatine monohydrate (**creatine**; Sigma-Aldrich), and phosphocreatine (Sigma-Aldrich) were all prepared to achieve a common 1 mM concentration. The solutions were likewise prepared in the standard method for their select molarity. Osmolarities were calculated for all solutions using the formula mOsm= Φ n C, where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration (mM; Table b).

Injection Procedure

Injections were performed with an AviTech Intellilab^{TM} Single Egg Injector, in order to reflect commercial application. Eggs were injected through the air cell. A blunt headed needle with a bore diameter (i.d.) of 0.6 mm (0.023 inches) was used in order to ensure that no injections were intramuscular. The total length of the needle was 18.4 mm

(7.25 inches), which provided an approximate 2.4 mm (0.98 inches) injection depth from the top of the large end of the egg. To ensure that the material was being delivered into the amnion, an intralab validation test was performed using a water soluble dye that was injected at Day 16 of incubation. The test confirmed that solution was being injected into the amnion. On Day 16 of development, eggs were weighed and randomly assigned to an injection treatment group prior to injection. In order to avoid crossover contamination of solutions, all replicate groups of eggs belonging to a particular treatment were injected with their corresponding solution before switching to another treatment. Solutions were first drawn into a syringe and the syringe was then attached to the machine. Three mL of each solution were pumped into the machine prior to injection in order to prime the system. Injection volumes of 200 μL were used in trials 1, 2, and 4, and in trial 3, the volumes were made as previously described (200, 800, 1000, and 2000 μL). The standard error for injection was $\pm$ 0.1 μL / 100 μL. Eggs were injected individually and the needle was disinfected after each injection. Once the treatment groups were completed, they were placed back into the incubator in their corresponding tray and level.

Data collection

Egg weights at set and at Day 16 (time of injection) were recorded. Egg weights at these times were determined in order to statistically correct for any possible treatment bias and to ensure that weight loss was consistent. On Day 18 of development eggs were weighed, arranged numerically, and then broken out. Embryos were subsequently

removed, and yolk sacs and embryos were weighed separately. Any developmental or positional abnormalities were noted, as well as any obvious physiological anomalies or contamination. Any data from contaminated eggs was not included in the final data analysis. Embryos and yolks were placed into a drying oven until there was no more noted weight loss (at least 2 weeks), and then dry weights of each were recorded. In trials 1, 2, 3, and 4, all late stage embryos (Days 16 to 18) that showed no evidence of contamination were considered for analysis. The following parameters were evaluated in all 4 trials: set egg weight; percent incubational egg weight loss (0-18 d); and relative embryo body weight, relative yolk sac weight, percent embryo moisture, and percent yolk sac moisture at Day 18 of incubation. Also, all trials were evaluated in terms of percent embryo mortality at the early-, mid-, and late- stages were also evaluated in all trials.

Statistical Analysis

A completely randomized block experimental design, with incubation tray level as a block, was employed. Individual eggs were considered as sub-samples within each replicate treatment group. Individual trial data were analyzed separately. A non-injected control treatment and a standard saline (117 mM) –injected treatment were included among the treatment groups examined. Means were compared in the event of significant global effects (Steel and Torrie,1980). Global effect means were considered significant at $P \leq 0.10$ and treatment mean separations were considered significant at with $P \leq 0.05$. All data were analyzed using the GLM procedure of SAS software, version 9.1 (SAS

Institute, 2003), that contained a random statement to account for sub-sampling. In the event of statistical set egg weight differences, set egg weight was assigned as a covariate in the analysis.

Results

In trial 1, set egg weight, percent incubational egg weight loss, relative embryo body weight, percent embryo moisture, percent yolk sac moisture, and mortality were not significantly affected by treatment. However, it was found that treatment did affect relative yolk sac weight ($P \leq 0.10$; Figure 3.1). Except for the 130 mM Na Ace treatment, relative yolk sac weight was statistically increased by all injected solutions in comparison to non-injected controls. Furthermore, saline significantly increased relative yolk sac weights compared with 118 and 130 mM Na Ace, and 0.5 and 1.5 mM NaH_2PO_4. Upon comparing treatments containing the same solute and within a close range of molarity, no statistically significant differences were observed. In trial 2, there were no significant treatment effects for set egg weight, percent incubational egg weight loss, relative embryo body weight, percent embryo moisture, relative yolk sac weight, percent yolk sac moisture, or mortality.

In trial 3, there were no significant affects due to treatment for set egg weight, relative embryo body weight, relative yolk sac weight, percent embryo moisture, percent

yolk sac moisture, or mortality. However, there was a significant treatment effect on percent egg weight loss ($P \leq 0.05$; Figure 3.2). The addition of 200 µL of saline, and 1000 and 2000 µL volumes of KCl or saline significantly decreased percent incubational egg weight loss below that of the non-injected control group. Nevertheless, the 200 µL and 800 µL volumes of KCl and the 200 µL volumes of saline did not produce an effect that was statistically different from that of the control group. Upon comparing treatments containing the same solute and within a close range of molarity, no statistically significant differences were noted, except for a significant difference between the 800 and 2000 µL volumes of saline. The 2000 µL volume reduced percent incubational egg weight loss in comparison to that of the 800 µL volume of saline. In trial 3, there was also a significant effect due to treatment on relative embryo body weight ($P \leq 0.05$; Figure 3.3). It was found that relative embryo body weight in eggs injected with KCl at the 1000 µL volume and saline at the 200, 800, and 1000 µL volumes were statistically lower than the control. When comparing treatments containing the same solute and within a close range of molarity, relative Day 18 embryo body weight was lower for the 1000 µL compared to the 200 µL KCl volume, and was higher for the 2000 µL compared to the 800 µL volume of saline.

In trial 4, there were no significant treatment effects on set egg weight, percent egg weight loss, relative embryo body weight, percent embryo moisture, relative yolk sac weight, percent yolk sac moisture, or mortality. However, there was a significant treatment effect on relative yolk sac weight ($P \leq 0.05$; Figure 3.4). No significant

differences occurred between all injected treatments and the non injected control. However, all were significantly higher than the saline injected control.

All measurements found to be significant were normalized using calculated miliosmolality (**mOSm**). The mOsm for the solutions was calculated using the formula mOsm= Φ n C; where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration. These figures and the numbers used to achieve them are reported in the Appendix.

Discussion

The results of trial 1 suggest that most of the solution types and concentrations tested increased Day 18 relative yolk sac weight in comparison to controls. The exceptions to this were 118 and 130 mM NaAce, and 0.5 and 1.5 mM NaH_2PO_4. The KCl at all concentrations (4.0, 4.5, 5.0, and 5.5 mM) tested increased relative yolk sac weight. Upon comparing the effects of the various concentrations within the NaAce and NaH_2PO_4 solutions, it was found that concentration had no effect. However, upon normalization of the data with calculated mOsm it was found that NaH_2PO_4 is more affected by mOsm than the KCl or NaAce (Figure 3.2b). However, unlike KCl, in the concentration ranges tested, both NaAce and NaH_2PO_4 lack the effectiveness to increase relative yolk sac weight. Therefore, it is possible that KCl concentrations between 4.0 and 5.5 mM have the potential to effectively decrease the rate of yolk sac absorption from

16 to 18 d of incubation. The possible ramifications of this effect on hatchability and posthatch growth are considered in subsequent trials.

In trial 3, the reduction of 0 to 18 d percent incubational egg weight loss through the use of 200, 1000, and 2000 µL volumes of saline (117 mM) or 1000 and 2000 µL volumes of KCl (5.5 mM) suggest that the higher volumes of both solutions are effective in reducing incubational egg weight loss. This is further supported by the fact that the 2000 µL volume of saline reduced egg weight loss in comparison to the 800 µL volume. Added saline or KCl solutions at these volumes apparently significantly replaced the moisture that is lost through normal egg shell conductive processes during incubation. However, because eggs must lose on the order of 12 to 15% of their set egg weight prior to pipping (Ar, 1991), the possible effects of solution replacement on time of hatch and hatchability must be considered. It was noted that upon normalization for mOsm that with a constant molarity KCl has a more substantial volume effect than the volume effect of NaCl (Figure 3.2b, 3.3b). What is also noted is that in both percent egg weight loss and relative embryo body weight the effects are similar and relative to each other. These results support an exploration into the effects of solution volumes larger than the commonly used injection volumes in other trials of this study.

In trial 4, the injection of 117 mM saline at Day 16 significantly reduced Day 18 relative yolk sac weight in comparison to the non-injected control group, as well as the treatments in which 1 mM concentrations of caffeine, theophylline, creatine or phosphocreatine were used. This would suggest that 117 mM saline itself caused a

reduction in relative yolk sac weight because of the comparatively higher relative yolk sac weight in the control group. Because of the opposite effect of the 117 mM saline in comparison to controls was noted in trial 1, further controlled studies should be conducted to confirm this specific effect on the yolk sac. Furthermore, the results also indicate that none of the stimulants tested had a negative influence on relative yolk sac weight. When normalization of the solutions, by mOsm, was applied it was found that the stimulant solutions maintained their original relativity to the control and to the original measurements. These stimulants might, therefore, have no effect on yolk utilization by the embryo between Days 16 and 18 of incubation. If these stimulants were used for purposes of stimulating the hatching process they might then have no subsequent affect on yolk energy reserves.

References

Devlin, T.M. 1997. Textbook of Biochemistry with Clinical Correlations. Wiley and Sons Printing. New York, New York. 206-208, 688-689.

Foye. O.T., Z. Uni, P.R. Ferket. 2006. Effects of In Ovo Feeding Egg White Protein, β-Hydroxy-β-Methylbutyrate, and Carbohydrates on Glycogen Status and Nenonatal Growth of Turkeys. Poultry Sci. 85:1185-1192.

Islam, S., M. Abley, N.H. Alam, F. Doussou, A.K.A. Chowdhury, J.F. Desjeux. 2004. Water and Electrolyte Salvage in an Animal Model of Dehydration and Malnutrition. Journal of Pediatric Gastroenterology and Nutrition. 38: 27-33.

Ohata, Y., M.T. Kidd, T. Ishibashi. 2001. Embryo Growth and Amino Acid Concentration Profiles on Broiler Breeder Eggs, Embryos, and Chicks After In Ovo Administration of Amino Acids. Poultry Sci. 80:1420-1436.
York.

Siegel, G.J., B.W. Agranoff, R.W. Albers, S.K. Fisher, M.D. Uhler. 1999. Basic Neurochemistry Molecular, Cellular, and Medical Aspects, 6th Edition. Lippincott, Williams and Wilkins Publisher. Ch 17.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed. McGraw-Hill, New York, NY.

Uni, Z., P.R. Ferket, E. Tako, O. Kedar. 2005. In ovo Feeding Improves Energy Status of Late-Term Chick Embryos. Poultry Sci. 84: 764-770.

Voet, D. and J.G. Voet. 2004. Biochemistry, 3rd edition. Wiley and Sons Printing. New York, New York.

Table 3.1a Solutions used in trial 1 including pH.

Trial 1		
Solution	Molarity (mM)	pH
Sodium Acetate ($CH_3COONa \cdot 3H_2O$)	110	9.41
Sodium Acetate ($CH_3COONa \cdot 3H_2O$)	118	9.31
Sodium Acetate ($CH_3COONa \cdot 3H_2O$)	125	9.22
Sodium Acetate ($CH_3COONa \cdot 3H_2O$)	130	8.67
Sodium Phosphate (NaH_2PO_4)	0.5	7.91
Sodium Phosphate (NaH_2PO_4)	1.0	7.36
Sodium Phosphate (NaH_2PO_4)	1.5	7.06
Sodium Phosphate (NaH_2PO_4)	2.0	7.02
Potassium Chloride (KCl)	4.0	8.96
Potassium Chloride (KCl)	4.5	9.34
Potassium Chloride (KCl)	5.0	9.14
Potassium Chloride (KCl)	5.5	9.17

Table 3.2 Solutions used in trial 2 including pH.

Trial 2		
Solution	Molarity (mM)	pH
CEN [Sucrose]	73.0	8.19
CEN [Sucrose]	31.6	8.71
CEN [Sucrose]	0.2	8.96
CEN [Sucrose]	9.0×10^{-3}	9.18
Caffeine ($C_8H_{10}N_4O_2$)	1.0	9.24
Caffeine ($C_8H_{10}N_4O_2$)	1.0×10^{-3}	9.21
Caffeine ($C_8H_{10}N_4O_2$)	1.0×10^{-6}	9.23
Caffeine ($C_8H_{10}N_4O_2$)	1.0×10^{-9}	9.25
Tripotassium Citrate ($HOC(COOK)(CH_2COOK)_2 \cdot H_2O$)	100	8.91
Tripotassium Citrate ($HOC(COOK)(CH_2COOK)_2 \cdot H_2O$)	5.0	9.09
Tripotassium Citrate ($HOC(COOK)(CH_2COOK)_2 \cdot H_2O$)	0.2	9.09
Tripotassium Citrate ($HOC(COOK)(CH_2COOK)_2 \cdot H_2O$)	1.2×10^{-2}	9.08

Table 3.3 Solutions used in trial 3 including pH.

Trial 3		
Solution	Molarity (mM)	pH
Potassium Chloride (KCl)	5.5	9.17
Sodium Chloride (NaCl)	117	8.32

Table 3.4a Solutions used in trial 4 including pH.

Trial 4		
Solution	Molarity (mM)	pH
Caffeine ($C_8H_{10}N_4O_2$)	1.0	9.26
Theophylline ($C_7H_8N_4O_2$)	1.0	9.27
Creatine ($C_4H_9N_3O_2 \cdot H_2O$)	1.0	9.13
Phosphocreatine ($C_4H_8N_3Na_2O_5P.4H_2O$)	1.0	8.59

Table 3.5 Carbohydrate/electrolyte solution (CEN) constitutive elements with mM concentrations as studied in trial 2.

Solution Strength (mM)				
	CEN, full strength	CEN [1:20]	CEN [1:40]	CEN [1:60]
Potassium Chloride[1]	22.0	1.1	0.05	$2.7x10^{-3}$
Tripotassium Citrate[2]	1.0	$5.0x10^{-2}$	$2.5x10^{-3}$	$1.2x10^{-5}$
Magnesium Chloride[1]	1.5	$7.0x10^{-2}$	$3.7x10^{-3}$	$1.8x10^{-4}$
Zinc Acetate[2]	0.3	$2.0x10^{-2}$	$7.0x10^{-4}$	$3.7x10^{-5}$
Copper (II) Sulfate[2]	4.0×10^{-2}	$1.7x10^{-3}$	$8.7x10^{-6}$	$4.4x10^{-6}$
Sodium Citrate[2]	9.9	0.5	$2.0x10^{-2}$	$1.2x10^{-3}$
Sodium Chloride[2]	59.9	3.0	0.2	$7.5x10^{-3}$
Glucose[1]	111.0	5.5	0.3	$1.4x10^{-2}$
Sucrose[2]	73.0	3.6	0.2	$9.1x10^{-4}$

[1] Fisher Scientific; [2]Sigma-Aldrich

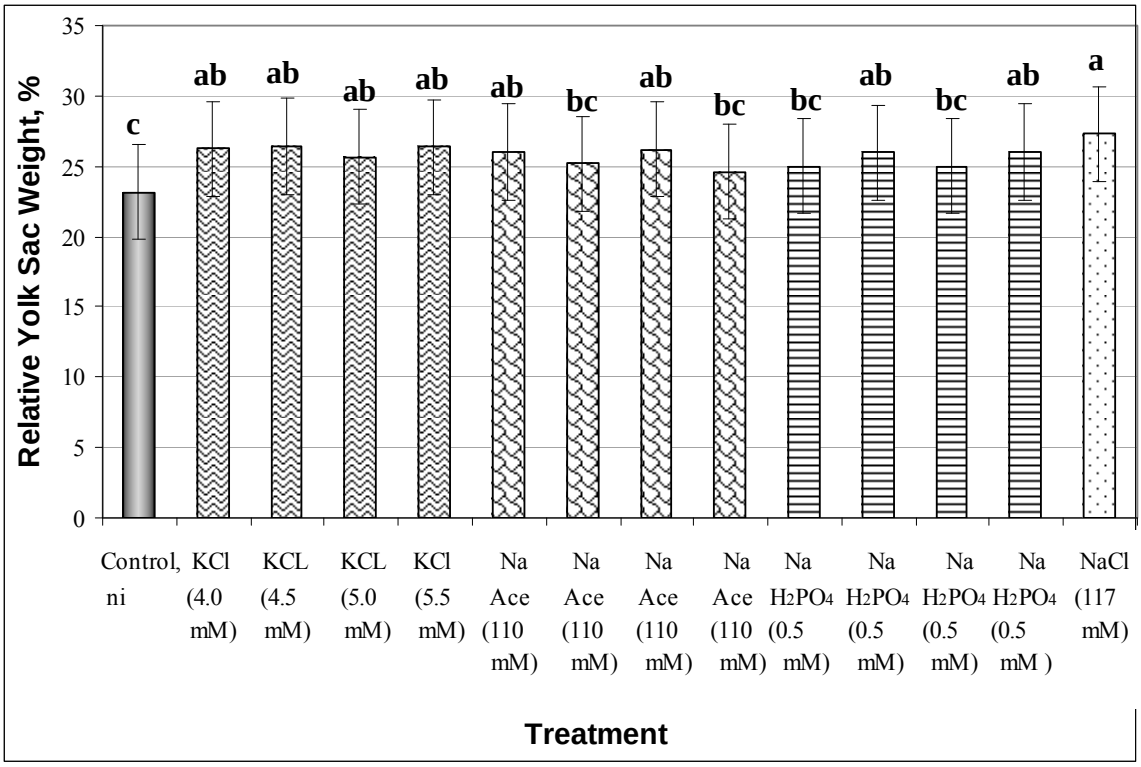

FIGURE 3.1a. Relative yolk sac weight of Day 18 broiler embryos ((wet yolk sac- dry yolk sac)/ wet yolk sac) in the following treatment groups: potassium chloride (KCl) at 4.0, 4.5, 5.0, and 5.5 mM; sodium acetate (Na Acetate) at 110, 118, 125, and 130 mM; sodium dihydrogenphosphate (NaH_2PO_4) at 0.5, 1.0, 1.5, and 2.0 mM; saline at 117 mM, and a non-injected control (control, ni) in trial 1.[1]

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

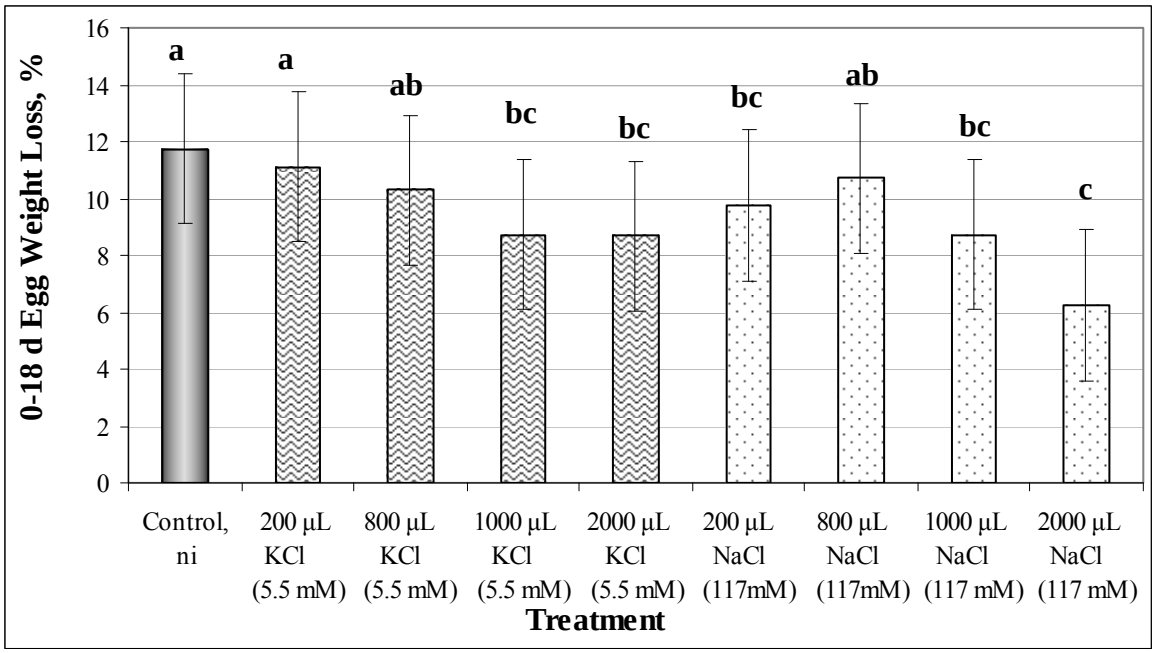

FIGURE 3.2a. Percent 0- 18 d incubational egg weight loss (0-18 d water loss(g)/ set egg weight (g)) in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control, ni) in trial 3[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 2 replicate units used for calculation of means.

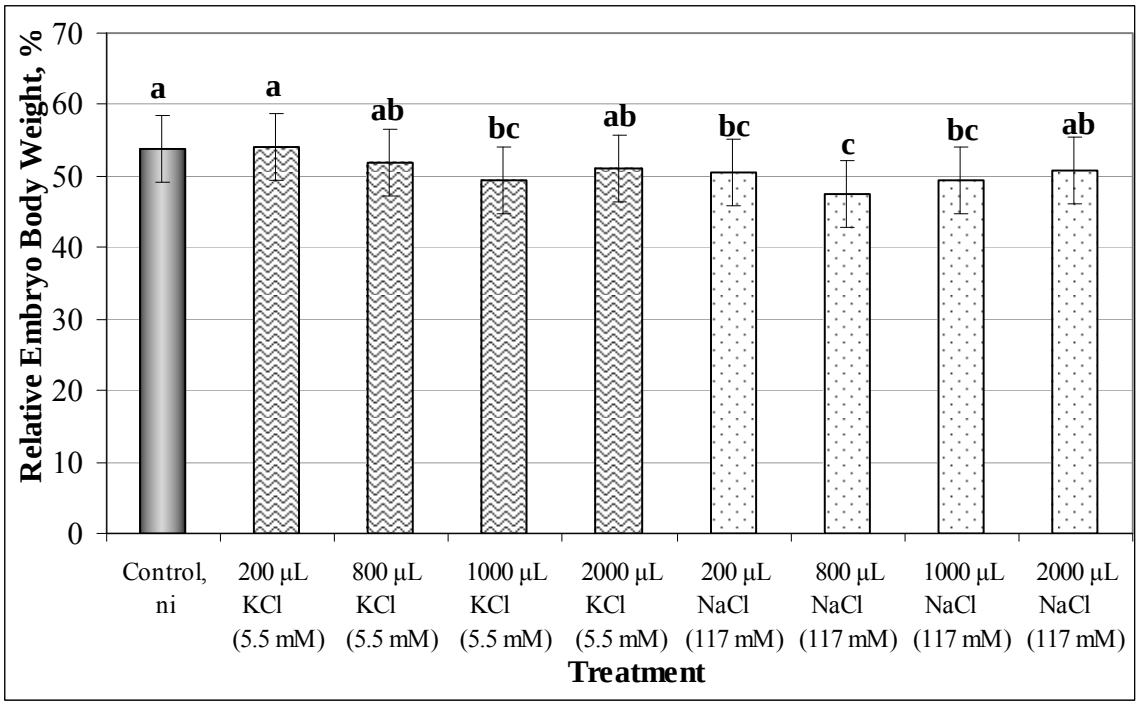

FIGURE 3.3a. Relative embryo body weights of Day18 broiler embryos (wet embryo (g)/dry embryo (g)) in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control) in trial 3[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 2 replicate units used for calculation of means.

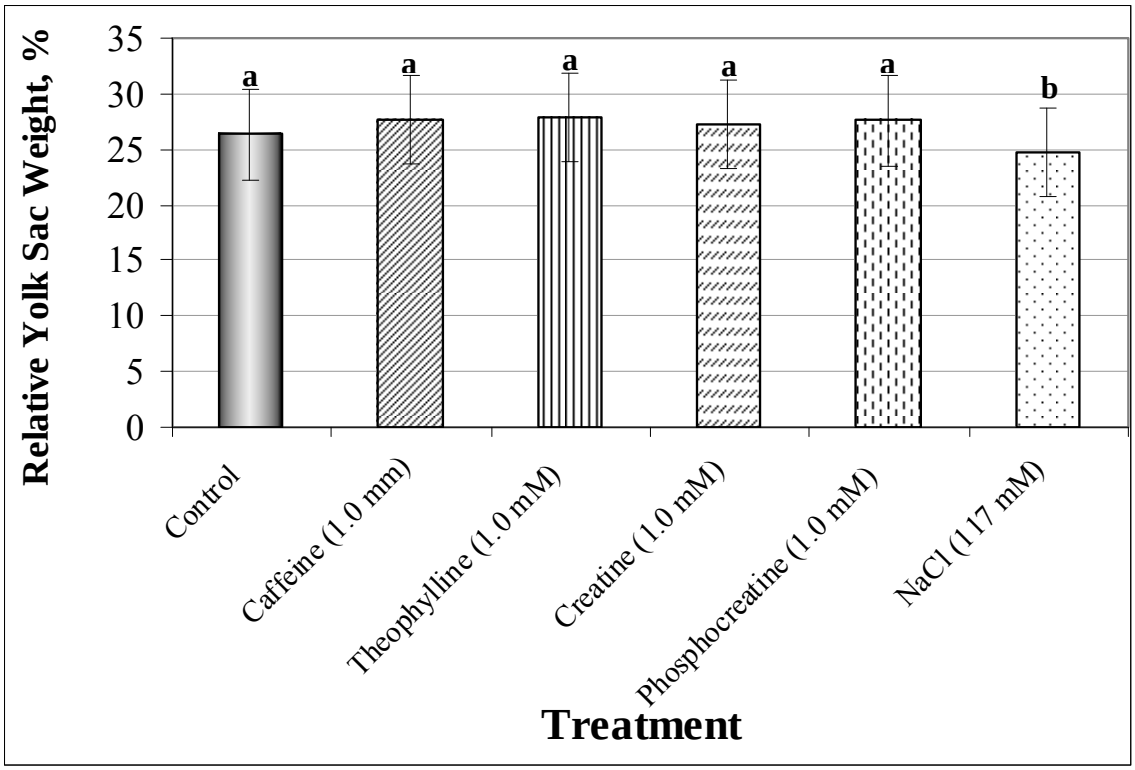

FIGURE 3.4a. Relative yolk sac weight of Day 18 broiler embryos (wet yolk sac (g)/ D 18 egg weight (g)) in the following treatment groups: 1 mM caffeine, theophylline, creatine and phosphocreatine; 117 mM saline; and a non-injected control (control, ni) in trial 4[1].

[a-b] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 2 replicate units used for calculation of means.

CHAPTER IV

EFFECTS OF COMBINATORIAL ELECTROLYTE WITH CARBOHYDRATE OR ELECTROLYTE AND STIMULANT SOLUTIONS ON EMBRYOGENESIS IN BROILER HATCHING EGGS

Abstract

Effects of automated *in ovo* injection of 5.5 mM potassium chloride in conjunction with a carbohydrate, a stimulant or another salt on the livability and growth of broiler embryos were investigated. Two trials were conducted separately. Solutions were injected into the amnion of embryos at Day 16 of incubation. Embryo mortality; relative embryo weight and moisture content; relative dry embryo weight; and relative yolk sac weight and yolk moisture content were evaluated on Day 18 of incubation in each trial. Trial 1 was conducted to determine the effectiveness of a 5.5 mM solution of potassium chloride as a carrier solution for various compounds previously described. The 5.5 mM potassium chloride solution was tested in combination with a carbohydrate/electrolyte solution, tripotassium citrate, and sodium dihydrogen monophosphate. In trial 2, 5.5 mM potassium chloride solutions were also tested in combination with the addition of 1mM caffeine, theophylline, creatine monohydrate, and L-arginine. None of the injected solutions had a significant effect on embryo mortality. In trial 1, relative embryo body weight was significantly reduced by the

carbohydrate/electrolyte, sodium monophosphate, and tripotassium citrate solutions. In trial 1, saline and all injected solutions carried in potassium chloride (5.5 mM) increased embryo body moisture over that of the control. In trial 2, all 4 solutions had no effect of any kind on the parameters examined. In conclusion, the solutions tested have potential for use individually or in combination in the commercial injection of broiler hatching eggs, however variable effects on embryo body weight and moisture content may result.

Introduction

The embryo is protected by a shell, but it also has a limited nutrient supply because it is not attached to its mother by an umbilical cord. While the enclosed environment in which avian embryos grow provides the necessary reservoirs for embryogenesis, the one-time supply of these resources from the mother may become limiting under the pressures of commercial production. As the embryo develops, it utilizes, and eventually uses, all of its available sources of nutrition. Over the past few years, some researchers have utilized *in ovo* injection in an effort to increase chick hatch weight and bird size at the time of processing. It has been shown by Uni *et al*. (2005) and Foye *et al*. (2006) that the injection of nutritional supplements benefit post hatch growth and the weight gain of birds. The broiler embryos (Ross) examined by Uni *et al.* (2005) showed no significant gain after *in ovo* feeding on Days 19 and 20 of embryonic development. Herein, attempts were made to demonstrate the usefulness of the addition of electrolytes in the development of embryos between Days 16 and 18 of incubation. In the following experiments, the possible use of electrolyte solutions injected *in ovo* to

promote growth and development of the avian embryo were explored. Multiple electrolyte solutions, all with a 5.5 mM potassium chloride base, and a stimulant carried in 5.5 mM potassium chloride were evaluated.

In previous studies, the potential use of other physiological salt solutions was investigated and it was found that they could serve to increase relative yolk sac weight. This may serve to allow for the conservation of nutritive resources for the embryo. It is thought that this conservation of the yolk was due to the increase in moisture due to the injection of a salt electrolyte solution.

In trial 1, 5.5 mM potassium chloride was combined with a carbohydrate based solution, a stimulant, or another salt. Potassium chloride was selected due to its ability to retain its effectiveness within a 1 mM concentration. It is possible that potassium chloride may be useful to conserve the yolk sac without having an adverse effect on the body weight. It may be able to complement the effects of additional electrolyte salts and the addition of a stimulant. As shown in Chapter III, trial 1, a concentration of 4.0 to 5.5 mM of potassium chloride were equally effective. Therefore, potassium chlorides may serve as a non-toxic base, up to 5.5 mM. With regards to the carbohydrate/electrolyte solution there are several reports that indicate that increasing the potassium chloride concentration is not detrimental to the overall function of the solution (Islam *et al.,* 2004). Sodium phosphate ($NaH_2 \bullet PO_4$) has the potential of adding additional phosphate (PO_4) to the overall system, allowing the increase concentration and potential storage in high energy compounds. Tripotassium citrate has the possibility of providing additional

potassium to cellular function, and also citrate rather than a negative chloride ion. It can also serve as an acidity regulator and an antioxidant.

In trial 2, the effects of stimulants and high energy compounds carried in 5.5 mM potassium chloride was evaluated. Caffeine serves as both a muscle stimulator, and a central nervous system stimulator (Voet and Voet, 2005, Fredholm *et al.*, 1999). It is proposed that the *in ovo* injection of caffeine would stimulate cellular activity (which may promote growth) and may also stimulate muscle activity (which may aid in the pipping response). Theophylline was also tested because it is a product of caffeine catabolism (Devlin, 1997). There are certain cell types that respond better to theophylline than caffeine, and as such each form has a distinctive response in different cells (Devlin, 1997). To see which provided the best response in avian embryos both were tested. Creatine is a high energy storage compound found in many different cells (Devlin, 1997). However, phosphocreatine serves as the actual energy reservation molecule. It is possible that the addition of creatine to the salt solution may encourage the cells to load the creatine, causing more yolk utilization during incubation and reducing the "wasted" yolk. L-Arginine was evaluated for its potential as a glycolytic amino acid and has shown to be effective in turkeys (Foye *et al.*, 2006). Arginine, which was studied in trial 2, is a known glycolytic amino acid (Voet and Voet, 2004; Devlin, 1997). Glucose is important in electrolyte solutions as an electrolyte balancer, functioning by increasing water absorption (Rehrer, 2001; Snyder, 1991). Amino acids that serve glycolytic functions have proven useful in the place of glucose in some tested electrolyte solutions (Zhai, 2008; Lima, *et al.,* 2002).

Materials and Methods

Incubation

Broiler hatching eggs (Ross x Ross) were obtained from a commercial source (Peco Farms, Inc). All eggs used within a trial were taken from a common flock. Eggs used within different trials were taken from different flocks. However, all eggs were taken from flocks that were between 35 and 54 wk of age. All eggs were held for 3 to 4 d prior to setting. Set weights were taken for individual eggs and the eggs were arranged randomly in each of two incubator tray (experimental blocks). In each tray, all treatments were equally represented and representative replicate groups of eggs for each treatment were set at random. The number of eggs set within each treatment replicate group, and saline group, for trials 1 and 2, were 10, and 8, respectively. In both trials there were 10 eggs in the 2 control replicate groups. There were 2 individual treatment replicate units in trials 1 and 2. In trials 1 and 2, the individual replicate units were represented by 2 tray columns on one tray level. Eggs were incubated in a Jamesway AVN single stage incubator for all trials. Incubator dry bulb temperatures were set at 37.6°C, with a variable range of 37.5 to 37.7°C during incubation. Incubator wet bulb temperatures were maintained between 26.0º and 30.0º C (i.e. 83 and 86% relative humidity).

Preparation of Solutions

Solutions were prepared within 2 to 5 d of injection. In all trials, a 117 mM saline treatment was used as a standard and there was a non-injected control (**control**). In trials 1 and 2, autoclaved water was used to prepare solutions and then the solutions were filtered through a TRP© single use, gamma sterilized syringe filter (.22 μm) the day before injection. In trial 1, the following compounds and stimulants were carried in 5.5 mM potassium chloride (**KCl**; Fisher Scientific): carbohydrate/electrolyte (1:40; **CEN**), tripotassium citrate monohydrate (5.0 mM; **3K Cit**; Sigma-Aldrich), and sodium phosphate monobasic (1.0 mM; **NaH_2PO_4**; Sigma-Aldrich) were investigated. The concentrations of the compounds found in the CEN are listed in Table 4.3. In trial 1, the solutions were made similar to the method described previously (Chapter III, trial 2), where a dilution was utilized to reach the desired volume. Once the final 1L volume was attained then the correct amount of KCl was added to create a KCl 5.5 mM concentration. Solution pH was measured and recorded (Table 4.1). In trial 2, the efficacies of 1 mM caffeine (anhydrous; $C_8H_{10}N_4O_2$; Sigma-Aldrich), theophylline (anhydrous), creatine monohydrate (**Creatine**; $C_4H_9N_3O_2 \cdot H_2O$; Sigma-Aldrich), or L-arginine in solution with KCl (5.5 mM; Rexall, Inc.) were investigated. The solutions were prepared in the standard method for their select molarity, and again the correct amount of KCl was added to ensure a 5.5 mM concentration when the solution was brought to 1 L. Solution pH was measured (Table 4.2). Osmolarities were calculated for all solutions using the

formula mOsm= Φ n C, where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration (mM; Table b).

Injection Procedure

Injections were preformed with an AviTech Intellilab™ Single Egg Injector, in order to reflect commercial application. Eggs were injected through the air cell. A blunt headed needle with a bore diameter (i.d.) of 0.6 mm (0.023 inches) was used in order to ensure that no injections were intramuscular. The total length of the needle was 18.4 mm (7.25 inches), which provided an approximate 2.4 mm (0.98 inches) injection depth from the top of the large end of the egg. To ensure that the material was being delivered into the amnion, an intralab validation test was performed using a water soluble dye that was injected at Day16 of incubation. The test confirmed that solution was being injected into the amnion. On Day 16 of development, eggs were weighed and randomly assigned to an injection treatment group prior to injection. For all replicate groups of eggs, the solutions belonging to a particular treatment were injected with their corresponding solution before switching to another treatment in order to avoid crossover contamination. Solutions were first drawn into a syringe and the syringe was then attached to the machine. Three mL of each solution were pumped into the machine prior to injection in order to prime the system. Injection volumes of 200 μL were used in trials 1 and 2. The standard error for injection was ± 0.1 μL / 100 μL. Eggs were injected individually and the needle was

disinfected after each injection. Once the treatment groups were completed, they were placed back into the incubator in their corresponding tray and level.

Data collection

Egg weights at set and at Day 16 of incubation (time of injection) were recorded. Egg weights at these times were used to ensure that weight loss was consistent and to account for any possible set egg weight differences in the data analysis of subsequent observations. On Day 18 of development eggs were weighed, arranged numerically, and then broken out. Day 18 egg weight was used to calculate 0 to 18 d percent incubational egg weight loss. Embryos were subsequently removed. Yolk sacs and embryos were weighed separately. Any developmental or positional abnormalities were noted, as well as any obvious physiological anomalies or contamination. Any data from contaminated eggs was not included in the final data analysis. Embryos and yolks were placed into a drying oven until weight loss ceased (at least 2 wk), and then dry weights of each were recorded. In trials 1 and 2, all late stage embryos (Days 16 to 18) that lacked evidence of contamination were considered for analysis. The following parameters were evaluated in all trials: set egg weight, percent incubational egg weight loss (0 to 18 d); and relative embryo body weight, relative yolk sac weight, percent embryo moisture, and percent yolk sac moisture at Day 18 of incubation. Also, all trials were evaluated in terms of percent embryo mortality at the early-, mid-, and late- stages of development.

Statistical Analysis

A completely randomized block experimental design, with incubation trays used as a block, was employed. Individual eggs were considered as sub-samples within each replicate treatment group. Individual trial data were analyzed separately. A non-injected control treatment and a standard saline (117 mM) –injected treatment were included among the treatment groups examined. Least-squares means were compared in the event of significant global effects (Steel and Torrie,1980). Global effects were considered significant at $P \leq 0.10$ and differences among least-squares means were considered significant at with $P \leq 0.05$. All data were analyzed using the GLM procedure of SAS software, version 9.1 (SAS Institute, 2003), that contained a random statement to account for sub-sampling. In the event of statistical set egg weight differences, set egg weight was assigned as a covariate in the analysis.

Results

In trial 1, there were no significant treatment effects on set egg weight, relative yolk sac weight, percent yolk sac moisture, or mortality. There was a significant treatment effect on Day 0 to 18 percent incubational egg weight loss ($P \leq 0.10$; Figure

4.1). Percent egg weight loss was significantly lower in all groups receiving an injected solution in comparison to controls. However, there were no significant differences between those group injected with a solution. There was a significant treatment effect on relative embryo body weight ($P \leq 0.10$; Figure 4.2). Relative Day 18 embryo body weight was significantly higher in the control group compared to the CEN, NaH_2PO_4, and 3K Cit groups with the saline group intermediate. Furthermore, the NaH_2PO_4 group was significantly lower than the saline group with the CEN and 3K Cit groups intermediate. There was also a significant treatment effect on percent Day 18 embryo body moisture ($P \leq 0.10$; Figure 4.3). Embryo body moisture at Day 18 was significantly higher in all groups injected with a solution compared to controls.

In trial 2, there were no significant treatment effects on set egg weight, percent incubations egg weight loss (0 to 18 d), relative embryo body weight, percent embryo moisture, relative yolk sac weight, percent yolk sac moisture, or mortality.

All measurements found to be significant were normalized using calculated miliosmolality (**mOSm**). The mOsm for the solutions was calculated using the formula $mOsm = \Phi\ n\ C$; where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration. These figures and the numbers used to achieve them are reported in the Appendix.

Discussion

All the solutions injected in this trial (saline, CEN, NaH_2PO_4, and 3K Cit) significantly reduced percent 0 to 18 d incubational egg weight loss. This was associated with a significant increase in embryo moisture content. The water content of embryos has been shown by Ar (1991) to be inversely associated with incubation egg weight loss. Therefore, the results would suggest that the injection of 200 μL of all the solutions at their various concentrations tested are capable of significantly increasing body moisture. It would follow that the increase in body moisture was subsequent to the replacement of moisture levels in the egg that would have normally been lost from 16 to 18 d of incubation. However, the injection of an individual solution at a 200 μL volume was not previously shown to impact water loss during incubation (Chapter III).

It is also not clear as to why the injection saline reduced percent incubational egg weight loss to such an extent, as it has not performed in this manner in previous trials (Chapter III). The amnion serves to protect the embryo from desiccation and an influx of water to the amnion and embryo may occur from other compartments in the egg through a reversal of osmolarity or water potential between the embryo and the rest of the egg (Ar, 1991). Therefore, it is worth considering that the introduction of electrolytes and solutes may introduce a concentration gradient that would cause a shift in water movement to the embryonic tissues. However, as noted in Chapter III, the introduction of KCl at a similar concentration into the amnion did not have an effect on embryo

moisture content. Therefore, the creation of an osmotic gradient between the embryo and egg through the use of the solutions tested does not seem plausible.

Nevertheless, it is postulated that the additional metabolic activity induced by the CEN, NaH_2PO_4, and 3K Cit may have initiated the decrease in body weight. Despite the associated effects of all the injected solutions on incubation egg weight loss and embryo moisture content, relative embryo body weight at Day 18 was reduced by all the injected solutions except for saline. This would suggest that although moisture content was increased, other tissue contents may have been negatively affected by CEN, NaH_2PO_4, and 3K Cit, to cause an overall reduction in relative body weight. It might also be stated that although there was a loss in dry body matter, embryo weight was compensated for by the addition of moisture. The affected tissue components are unknown because of the limited tissue analysis. Further research should be conducted to determine how other tissue profiles are actually affected. This should include an analysis of body tissue fat, protein and carbohydrate. Despite natural increases in body tissue lipid and decrease in body tissue protein and moisture concentrations between 16 and 18 d of incubation (Peebles *et al.*, 1999), Romanoff and Romanoff (1967) reported that the most extensive use of lipids, via lipid hydrolysis and β-oxidation, begin after Day 15 of incubation. The higher percentage of water present in the embryo, therefore, may be a result of more extensive lipid hydrolysis, which was stimulated by the injected electrolyte solutions. It was noted that, after data was normalized using mOsm, that the effects across all significant measurements were similar and relative to each other (Figure 4.1b, 4.2b, 4.3b). This indicates a real response to these solutions in the embryo. Nevertheless,

although these embryonic tissue component changes might be exaggerated by the injection of these electrolyte solutions, they may be later corrected for through proper posthatch brooding. The possible effects on posthatch performance are further explained in the subsequent chapter (Chapter V).

In trial 2, no significant treatment effects were noted. This indicates that the addition of saline does not have a similar effect across trials. In Chapter III, trial 4, it was found that saline decreased yolk sac weight below that of controls. However, the stimulant treatments were not statistically different from each other or from the control.

References

Ar, A. 1991. Egg water movements during incubation. Avian Incubation. S.G. Tullett, ed. Butterworth-Heinemann Ltd., London, UK. 157-173.

Devlin, T.M. 1997. Textbook of Biochemistry with Clinical Correlations. Wiley and Sons Printing. New York, New York. 206-208, 688-689.

Foye. O.T., Z. Uni, P.R. Ferket. 2006. Effects of In Ovo Feeding Egg White Protein, β-Hydroxy-β-Methylbutyrate, and Carbohydrates on Glycogen Status and Nenonatal Growth of Turkeys. Poultry Sci. 85:1185-1192.

Lima, G.H.P. Carvalho, A.A. Figuerido, A.R. Gifoni, A.M. Soares, E.A.T. Silva, R.L. Guerrant. 2002. Effects of an Alanyl-Glutamine-Based Oral Rehydration and Nutrition Therapy Solution on Electrolyte and Water Adsorption in a Rat Model of Secretory Diarrhea Induced by Choera Toxin. Nutrition. 18:458-462.

Peebles, E. David, Lumu Li, Sherman L. Miller, Tomas Pansky, Sharon Whitmarsh, Mickey A. Latour, and Patrick D. Gerard, 1999. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poultry Sci. 78:1435-1442.

Romanoff, A. L. and A. J. Romanoff. 1967. Biochemistry of the Avian Embryo. Wiley and Sons Printing. New York, New York.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed. McGraw-Hill, New York, NY.

Uni, Z., P.R. Ferket, E. Tako, O. Kedar. 2005. In ovo Feeding Improves Energy Status of Late-Term Chick Embryos. Poultry Sci. 84: 764-770.

Voet, D. and J.G. Voet. 2004. Biochemistry, 3rd edition. Wiley and Sons Printing. New York, New York.

Zhai, W., S. Neuman, M.A. Latour, P.Y. Hester. 2008. The Effect of In Ovo Injection of L-Carnitine on Hatchability of White Leghorns. Poultry Science. 87:569-572.

Table 4.1a Solutions used in trial 1 including pH.

Trial 1		
Solution	Molarity (mM)	pH
CEN + KCL	0.18 + 5.5	8.98
3K Cit+ KCl	5.0 + 5.5	9.33
NaH_2PO_4 + KCl	1.0 + 5.5	7.37

Table 4.2 Solutions used in trial 2 including pH.

Trial 2		
Solution	Molarity (mM)	pH
Caffeine + KCL	1.0 + 5.5	9.08
Theophylline + KCl	1.0 + 5.5	9.43
Creatine + KCl	1.0 + 5.5	9.98
L-Arginine + KCl	1.0 + 5.5	9.33

Table 4.3 Carbohydrate/electrolyte solution (CEN) constitutive elements with mM concentrations as studied in trial 1.

Solution Strength (mM)	
	CEN [1:40]
Potassium Chloride	0.05
Tripotassium Citrate	2.5×10^{-3}
Magnesium Chloride	3.7×10^{-3}
Zinc Acetate	7.0×10^{-4}
Copper Sulfate	8.7×10^{-6}
Sodium Citrate	2.0×10^{-2}
Sodium Chloride	0.2
Glucose	0.3
Sucrose	0.2

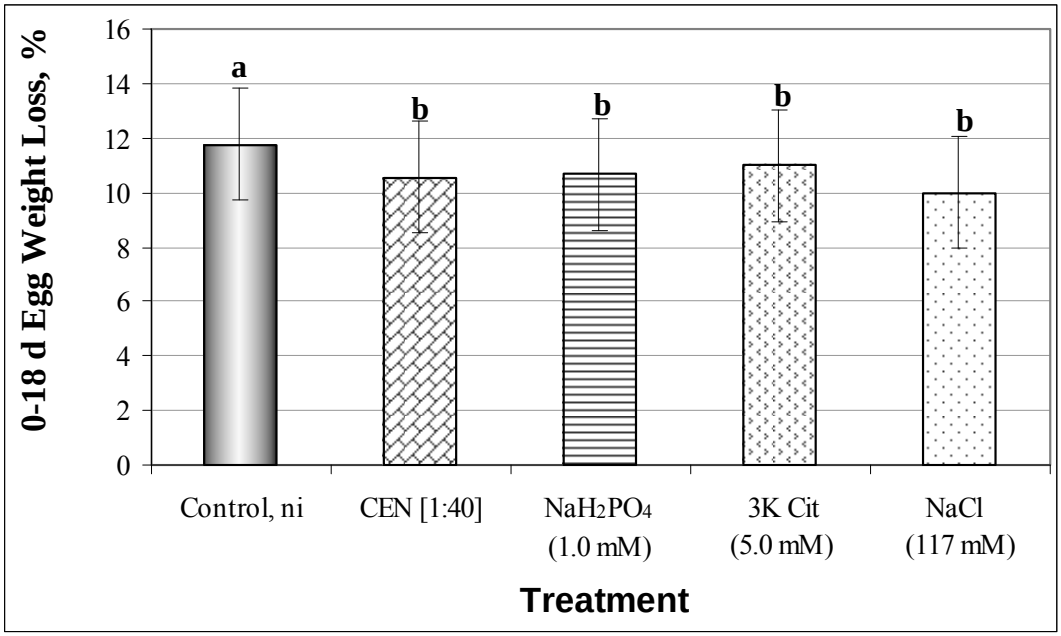

FIGURE 4.1a. Percent 0-18 d incubational egg weight loss (0-18 d water loss (g)/set egg weight (g)) in the following treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and non-injected control (control, ni) treatments in trial 1[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

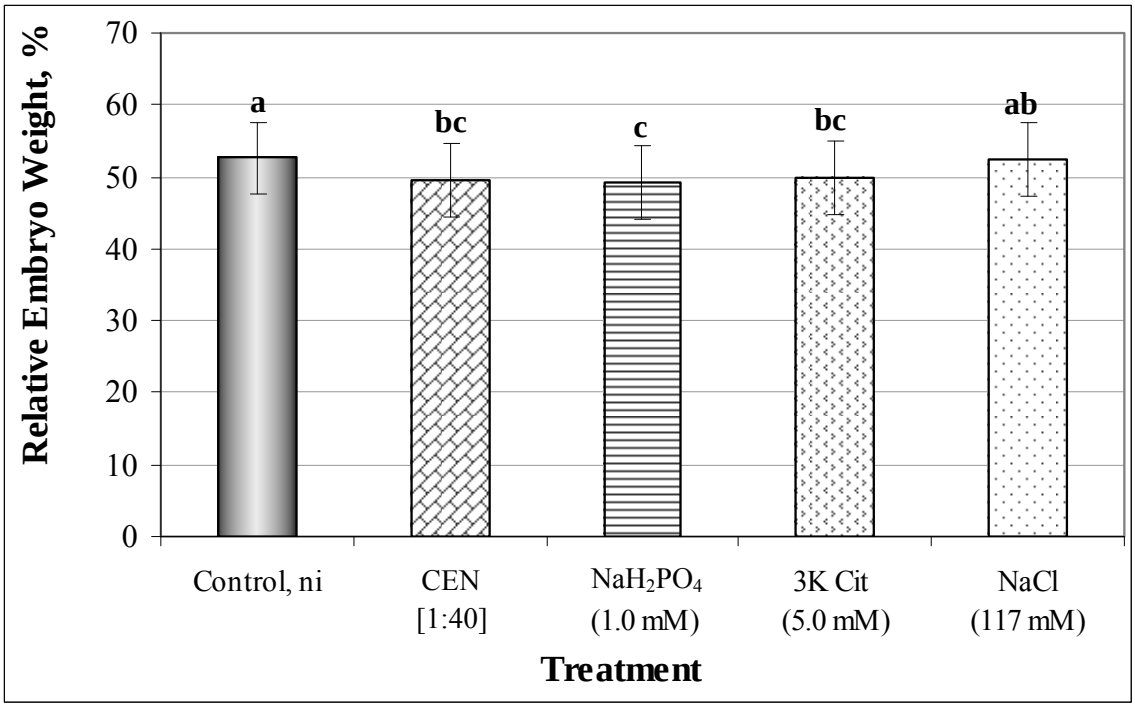

FIGURE 4.2a. Relative embryo body weight of Day 18 broiler embryos (wet embryo (g)/ D 18 egg weight (g)) in the following treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and non-injected control (control, ni) treatments in trial 1[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

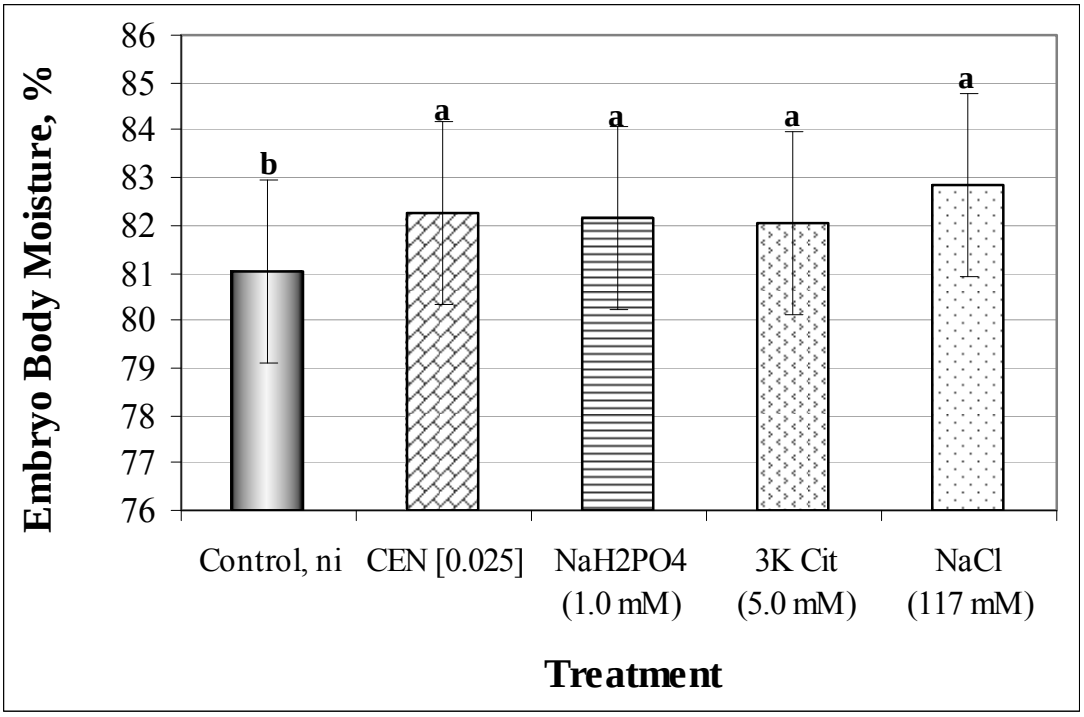

FIGURE 4.3a. Percent embryo body moisture of Day 18 broiler embryos ((wet embry-dry embryo) (g)/ wet embryo(g)) in the following treatment groups, where treatments were all carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and a non-injected control (control, ni) treatments in trial 1[1].

a-b means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

CHAPTER V

EFFECTS OF INJECTION OF COMBINATORIAL ELECTROLYTE SOLUTIONS ON THE HATCHABILITY AND GROWTH OF BROILERS FROM DAY 0 TO DAY 10 POST HATCH

Abstract

Effects of the automated *in ovo* injection of carbohydrate and stimulatory solutions, each carried in 5.5 mM potassium chloride, on the livability and growth of broiler chicks, through Day 10 post hatch, were investigated. Also, the effects of some solutions in conjunction with the stimulant theophylline, were subsequently investigated. Both trials were investigated separately. Solutions were injected into the amnion of embryos at Day 18 of incubation. In trial 1, the potential of 3 electrolyte (1 carbohydrate containing and 2 stimulant containing) solutions carried in potassium chloride to affect hatchability and post hatch chick performance was analyzed. The solutions used were a carbohydrate/electrolyte solution, tripotassium citrate, and creatine. In trial 2, the effectiveness of each of the aforementioned solutions from trial 1 in conjunction with the stimulant, theophylline, was investigated. In trial 1, 1.0 mM creatine significantly reduced chick weight at hatch compared to both non-injected control and 117 mM saline-injected groups, whereas in comparison to non-injected controls, relative yolk sac weight

at Day 3 was increased by 117 mM saline. In trial 2 there was an effect on Day 3 relative liver weight. Trial 2 was also the only trial in which late embryo mortality and was increased by all the injection treatments and hatch to Day 3 chick mortality was increased by the injection of 1.0 mM creatine in solution with 5.5 mM potassium chloride and 1.0 mM theophylline. However, the effects on mortality may have been exacerbated by the poor brooding conditions that chicks were subjected to in trial 2. Because of this the solutions tested are believed to have potential for use individually or in combination in the commercial injection of broiler hatching to promote subsequent hatchability and post hatch growth.

Introduction

The embryo is protected by a shell, but it also has a limited nutrient supply because it is not attached to its mother by an umbilical cord. While the enclosed environment in which avian embryos grow provides the necessary reservoirs for embryogenesis the one time supple of these resources from the mother may become limiting under the pressures of commercial production. As the embryo develops it utilizes, and eventually uses, all of its available sources of nutrition. Over the past few years, some researchers have utilized *in ovo* injection in an effort to increase chick hatch weight and bird size at the time of processing. It has been shown by Uni *et al*. (2005) and Foye *et al*. (2006) that the injection of nutritional supplements benefit post hatch growth and the weight gain of birds. The broiler embryos (Ross) examined by Uni *et al.* (2005) showed no significant gain after *in ovo* feeding on Days 19 and 20 of embryonic

development. Uni *et al.* (2005) also attributed a statistical growth in Ross birds after 10 d post hatch.

In previous trials of this study, it has been have shown that certain components of electrolyte solutions are not harmful in late avian embryogenesis and that there is the potential that these solutions could promote post hatch growth and development. Herein, the usefulness of these electrolyte solutions in the development of chicks 0 to 10 d is demonstrated. The benefits of the individual electrolyte component, as well as the added potentials when mixed, and combined with a stimulant were investigated. Attempts were made to demonstrate the usefulness of electrolyte solutions in the development of post hatch chicks from day of hatch to Day 10 post hatch. In the following experiments, the possible use of electrolyte solutions, some containing a stimulant, injected *in ovo* to promote growth and development of broilers were explored.

The solutions investigated may serve a multitude of purposes. In previous studies, the potential use of other individual salt solutions was investigated. Potassium chloride is a commonly known physiological salt, and has the potential to be used as, or more, effectively as saline. These properties are demonstrated through the cellular pump activity in cells, in that there is always a continuous effort to expel sodium and retain potassium intracellularly (Voet and Voet, 2004). Because of potassium chloride's effect on the yolk sac in previous chapters it is believed that the conservation of yolk will aid in the lipid to carbohydrate diet switch. The carbohydrate/electrolyte nutrient solution was the only complex solution that was tested. It was chosen because previous research using simple carbohydrates for *in ovo* application has shown them to be effective in promoting

chick development between 10 to 24 d (Uni *et al.*, 2005). Also, because the solution was originally used in catch-up feeding programs for malnourished children, they may be potentially used in avian embryos and to promote post hatch growth. It also may allow for a smoother transition from a lipid to a carbohydrate- based diet. The injection a complex and simply carbohydrate solution may allow the embryo time to adjust, in the same manner as a child goes from breast milk to toddler food before progressing to a more adult diet. Tripotassium citrate has the possibility of allowing additional potassium to cellular function, and also adding free citrate rather than the chloride ion. It can also serve as an acidity regulator and an antioxidant, and as such it continued to hold an interest for growout studies. The current trials were designed to examine the effects of solutions previously examined as to their effects in embryos, and determine their subsequent effects on post hatch chick growth and development. Also, to further determine any correlation between embryo effects and the chick effects due to these solutions.

In trial 1, the effects of combinatorial electrolyte solutions were evaluated. These solutions were carried in 5.5 mM potassium chloride: creatine, carbohydrate/electrolyte solution, and tripotassium citrate. These are the same solutions that were previous tested in the embryo (Chapter IV, trials 1 and 2) and were found to have a positive effect on embryonic processes and were considered to be solutions of interest.

In trial 2, effects of the combinatorial electrolyte solutions used in trial 1, with the addition of a stimulant, were evaluated. Caffeine serves as both a cellular stimulator, and a central nervous system stimulator. The effects of theophylline were explored

because it is a product of the breakdown of caffeine. There are certain cell types that respond better to theophylline than caffeine (Devlin, 1997). And in previous studies theophylline had shown a better numerical response than caffeine in embryos (Chapter III, trial 4). In order to determine if theophylline would have a stimulatory effect on the pipping muscle, it was tested in conjunction with the solutions from trial 1. Creatine is a high energy storage compound found in many different cells. The creatine may promote metabolism and storage of adenosine triphosphate (**ATP;** Voet and Voet, 2004, Clark, 1997), but the cells would have to load the creatine. This process may not be beneficial, since it would utilize the embryo's own nutrient and energy sources, but it may promote total usage of the yolk. In previous, studies phosphocreatine led to a numerically had a better response than creatine. However, creatine was chosen for these studies because it was more available and less expensive than phosphocreatine while maintaining an effect that was not statistically different from that of phosphocreatine.

Materials and Methods

Incubation

Broiler hatching eggs (Ross x Ross) were obtained from a commercial source (Peco Farms, Inc). All eggs used within a trial were taken from a common flock. Eggs used within different trials were taken from different flocks. However, all eggs were taken from flocks that were between 35 and 54 wk of age. All eggs were held for 3 to 4 d

prior to setting. Average set weights were taken for replicate groups and the eggs were arranged randomly in each incubator tray level (experimental block). On each incubator level, all treatments were equally represented and representative replicate groups of eggs for each treatment were set at random. The number of eggs set within each treatment replicate group for trials 1 and 2, were both 10. The number of individual treatment replicate units in trials 1 and 2, were both 4. In trials 1 and 2, the individual replicate units were represented by 4 tray levels in each of 1 tray column. Eggs were incubated in a Jamesway AVN single stage incubator for both trials. Incubator dry bulb temperatures were set at 37.6°C, with a variable range of 37.5 to 37.7°C during incubation. Incubator wet bulb temperatures were maintained between 26.0º and 30.0º C (i.e. 83 and 86% relative humidity).

Preparation of Solutions

Solutions were prepared within 2 to 5 d of injection. In all trials there was a 117 mM saline and a non-injected control (**control, ni**) group. Solutions were prepared in autoclaved water and then were filtered through a TRP© single use, gamma sterilized syringe filter (0.22 μm) the day before injection. Potassium chloride (5.5 mM; **KCl**; Fisher Scientific), was used as a carrier for the injected solutions tested in both trials. In trial 1, the following compounds and stimulants were tested: a carbohydrate/electrolyte solution (**CEN**) at a relative concentration of 1:40; tripotassium citrate monohydrate (**3K Cit**; $HOC(COOK)(CH_2COOK)_2 \cdot H_2O$; Sigma-Aldrich) at a concentration of 5 mM; and creatine monohydrate (**creatine**; $C_4H_9N_3O_2 \cdot H_2O$; Sigma-Aldrich) at a concentration of

1.0 mM. In trial 1 the solutions used were as described in Chapter IV, trial 1, where the correct amount of KCl was added to create a 1.0 L solution with a final volume that was 5.5 mM. Solution pH was measured for all test solutions (Figure 5.1). In Trial 2, theophylline (anhydrous; $C_7H_8N_4O_2$; Sigma-Aldrich) was added to each solution. The solutions were prepared as previously described for their select molarity, and again the correct amount of KCl and theophylline were added to reach endpoint concentrations of 5.5 mM and 1.0 mM, respectively, in 1.0 L of solution. Solution pH was measured for all test solutions (Table 5.2). Osmolarities were calculated for all solutions using the formula mOsm= Φ n C, where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration (mM; Table b).

Injection Procedure

Injections were preformed with an AviTech Intellilab™ Single Egg Injector, in order to reflect commercial application. Eggs were injected through the air cell. A blunt headed needle with a bore diameter (i.d.) of 0.6 mm (0.023 inches) was used in order to ensure that no injections were intramuscular. The total length of the needle was 18.4 mm (7.25 inches), which provided an approximate 2.4 mm (0.98 inches) injection depth from the top of the large end of the egg. The test confirmed that solution was being injected into the amnion. On Day 18 of development, eggs were weighed and randomly assigned to an injection treatment group prior to injection. In order to avoid crossover contamination all replicate groups of eggs belonging to a particular treatment were injected with their corresponding solution before switching to another treatment.

Solutions were first drawn into a syringe and the syringe was then attached to the machine. Three mL of each solution were pumped into the machine prior to injection in order to prime the system. Injection volumes of 200 µL were used in trials 1 and 2. The standard error for injection was $\pm$ 0.1 µL / 100 µL. Eggs were injected individually and the needle was disinfected after each injection. Once the treatment groups were completed, they were placed back into the incubator in their corresponding tray and level.

Growout

On Day 18 of development, after injection, the eggs were placed into hatching baskets in the Jamesway AVN single state incubator. Hatching was monitored every 12 h and number of total chicks in the basket at each interval was recorded (results not reported). On Day 21/ Day 0 the chicks were removed and tagged with a numerical/color code. Chicks were assigned to pens which corresponded to their respective incubational replicate unit. Therefore, all treatments were represented within each of 4 replicate pens. The chicks were placed into the prepared pens, again by their treatment block. After hatch the chicks were placed, by block, into pens in a house. There were four pens, one for each replicate block. Pens were old bedding on cement floors and were warmed prior to setting. All pens were on the same side of the house. Chicks were shown food and water and they were monitored closely in the first 24 h to ensure they were in ideal conditions. All chicks had *ad libitium* access to food and ground waterers during the 10 day growout. Brooding lights were applied, and for trial 1 the temperature records of the house were taken bi-daily (not reported). For trial 2 it was found that the house was not

maintaining proper temperature under the brooding lights. Both house and brooding temperatures were recorded, bi-daily, 2 to 10 d (not reported).

Animal Care

All experimental protocols were approved by the Institutional Animal Care and Use Committees at Mississippi State University. All birds were given *ad libitum* access to water and diet formulated to meet or exceed NRC (1994) recommendations in all experiments.

Data collection

Total weights and numbers of eggs for each treatment replicate group at set and Day 18 (day of injection) were recorded. Likewise, at Day 0 (hatch), Day 3, and Day 10 of posthatch age, total chick numbers and group weights were recorded for each treatment replicate group. Subsequent mean egg and chick weights were calculated for each replicate group within each treatment. Mean body weight gains between Days 0 and 3, 3 and 10, and 0 and 10 posthatch were also examined. At hatch the birds were renumbered according to their block and treatment groups. Chick hatch weights were recorded prior to the birds were placement in the growout facility. Any chicks that died in between sampling dates had dead weights recorded, and a necropsy was preformed to determine cause of death.

The first sampling was done on Day 3. Chicks, randomly selected the day before, were collected in the morning and brought from the house to the sampling site. Live

weights were taken from the chicks. Chicks were then sacrificed by cervical dislocation and their chest was opened to collect blood for chem panels (data not shown here). Then the liver was removed, weight, and a sample was placed into perchloric acid for later testing. The remaining liver was kept for moisture analysis. The residual yolk sac was removed, weighed, and kept for moisture analysis.

The last sampling was also done on Day 10. The remaining chicks were collected from the house and brought to the sampling site. Live weights were taken from all the chicks. Chicks were then sacrificed by cervical dislocation and their chest was opened to collect blood for chem. panels (data not shown here). The liver was removed, weighed, and a sample was placed into perchloric acid for later testing. The remaining liver was kept for moisture analysis.

Statistical Analysis

A completely randomized block experimental design was employed, with incubation tray levels and growout pens. Individual eggs or chicks were considered as sub-samples within each replicate treatment group. Individual trial data were analyzed separately. A control treatment and a saline (117 mM) –injected treatment were included among the treatment groups examined. Means were compared in the event of significant global effects (Steel and Torrie,1980). Global effect means were considered significant at $P \leq 0.10$ and treatment mean separations were considered significant at with $P \leq 0.05$. All data were analyzed using the GLM procedure of SAS software, version 9.1 (SAS Institute, 2003), that contained a random statement to account for sub-sampling. In the

event of statistical set egg weight differences, set egg weight was assigned as a covariate in the analysis.

Results

In trial 1, there were no significant treatment effects on set egg weight; Day 18 injection egg weight; rate of hatch; chick hatch weight (individual and treatment replicate mean); Day 3 and 10 treatment replicate pen weight; Day 3 and 10 live necropsy weight; Day 3 relative yolk sac weight; Day 3 and 10 relative liver weight; 0 (hatch) to 3d, 0 (hatch) to 10 d, and 3 to 10 d body weight gain. There was also no significant effect on mortality in embryos or post hatch chicks. However, there was a significant treatment effect on mean hatch chick weight, ($P \leq 0.10$; Figure 5.1). Hatch chick weight was significantly higher in the saline group compared with the control. Hatch chick weight in the creatine treatment was significantly lower than the controls with the CEN and 3K Cit intermediate. There was also a significant effect on the relative yolk sac weight of Day 3 chicks ($P \leq 0.05$; Figure 5.2). Relative yolk sac weight was higher in the saline-injected group compared with the control, CEN and 3K Cit groups, with the creatine group intermediate. Furthermore the creatine treatment was significantly higher than the CEN treatment, with the control and 3K Cit treatments intermediate.

In trial 2, there were no significant treatment effects on set egg weight; Day18 injection egg weight; rate of hatch; chick hatch weight (individual and treatment replicate mean); Days 3 and 10 treatment replicate pen weights; Days 3 and 10 live necropsy weight; Day 3 relative yolk sac weights; Day 3 and 10 relative liver weights; 0 (hatch) to

3 d, 0 (hatch) to 10 d, and 3 to 10 d body weight gains. However, there was a significant effect ($P \leq 0.05$; Fig 5.3) on Day 3 relative liver weight. Relative liver weight of Day 3 chicks was higher in controls compared to the 3K Cit group, with the CEN and creatine groups intermediates. There was no significant effect on early- or mid- mortality in embryos. However, a significant effect on percent late dead mortality was found in the residue hatch analysis ($P \leq 0.05$; Figure 5.4). All injection treatments resulted in a statistically higher percent late dead mortality than the controls. A significant treatment effect was also seen for chick mortality between Day 0 (hatch) and Day 3 ($P \leq 0.05$; Figure 5.5). The creatine injection treatment caused the highest cumulative posthatch mortality than did the control, CEN and 3K Cit treatments, with the saline treatment intermediate.

All measurements found to be significant were normalized using calculated miliosmolality (**mOSm**). The mOsm for the solutions was calculated using the formula mOsm= Φ n C; where Φ was set equal to 0.95 disassociation, n is the number of dissociated ions, and C is the concentration. These figures and the numbers used to achieve them are reported in the Appendix.

Discussion

Although chick hatch weight was depressed in trial 1, it has been shown in previous trials that this decrease in weight is often marked by an increase in percent body moisture (Chapter IV, trial 1). In these previous studies, when embryo body weight was significantly depressed below that of the control, it was associated with a significant

increase in percent body moisture over that of the control (Chapter IV, trial 1). It was discussed that this loss in body weight may be attributed to the embryo beginning to extensively utilize lipids during the late stages of growth (Romanoff and Romanoff, 1967). A continued increase in moisture levels in these birds allowed for a normalization of body weight to that of control levels by Day 3 post hatch. This normalization was also accompanied by an increase in residual yolk by Day 3 in the saline injected group in comparison to controls. However, it is noticeable that the CEN solution resulted in a numerically lower residual yolk weight in comparison to controls. Considering the constituents of this solution, is also notable that many of them serve as metabolic intermediaries that are designed to promote body moisture and uptake of water (Lima *et al*, 2002; Rehrer, 2001). The addition of theophylline in trial 2 appeared to ameliorate the positive moisture effect, as it did in Chapter IV, trial 2, when it was combined with a salt. It was noted that when the data was normalized, using mOsm, that the effects between trial 1 and 2 were similar and relative, despite the addition and theophylline and the supposed amelioration of the positive moisture effect (Figure 5.1b, 5.2b, 5.3b).

Although there was no statistically significant effect due to creatine, creatine did have a numerically positive influence on growth. The creatine treatment caused birds to gain 10 g more in body weight than the controls, and at Day 10, the creatine treatment group outweighed the controls by 9 g.

In trial 2, the control birds had the highest relative liver weight, in comparison to the saline and 3KCit birds. It is unknown in trial 1 why there were yolk sac responses to treatment, whereas in trial 2 there was an effect on relative liver weight. It has been

shown by Nobel and Ogunyemi (1989) that growth of the liver after hatching was associated with a substantial accumulation of fat from that present in the embryonic chick reserves, and that the changes noted in the lipid and fatty acid composition of the liver were indicative of its rapidly altered role in lipid metabolism of the new chick. It is postulated that because of the poor brooding environment in trial 2 (from 0 to 3 d posthatch) that many of the chicks relied heavily on their yolk reserves, thus possibly compromising liver energy reserves in response to a lower feed intake. Upon review of the necropsy reports, it was found that all chicks were found with little to no food in the crop, and that they exhibited swollen kidneys, increased renal ureates, and some degree of breast muscle regression. It was the opinion of the veterinarian on site that increased mortality was due to the effects of the cold temperature and poor brooding.

There was also a significant effect on percent late dead mortality in the residual hatch; however, upon examination of the data, it was found that there were no late dead mortalities in the control group. All injection treatments resulted in a significantly higher percent late dead mortality than the controls. However, because there was also a significant effect among replicate groups within treatment groups, it is suggested that positional effects within the incubator may have been a confounding factor. The creatine treatment resulted in a significant increase in 0 to 3 d cumulative chick mortality compared to controls. However, there is no research in the literature to support a correlation between exogenous creatine supplementation and increased mortality. The CEN treatment, unlike creatine, did not adversely effect mortality. It is, therefore, possible that the ability of the CEN to promote hydration during stressful periods (Islam

et al., 2004) may have mitigated the chick's response to the poor brooding conditions and thereby improve livability. It was also noted that when the data was normalized to mOsm that the previously seen relative effects were no longer present. This is believed to strengthen the argument that the increased mortality in late term embryos and chick mortality is due to additional factors.

References

Clark, J.F. 1997. Creatine and Phosphocreatine: A Review of Their Use in Exercise and Sport. Journal of Athletic Training. 32: 45-51.

Devlin, T.M. 1997. Textbook of Biochemistry with Clinical Correlations. Wiley and Sons Printing. New York, New York. 206-208, 688-689.

Foye. O.T., Z. Uni, P.R. Ferket. 2006. Effects of In Ovo Feeding Egg White Protein, β-Hydroxy-β-Methylbutyrate, and Carbohydrates on Glycogen Status and Nenonatal Growth of Turkeys. Poultry Sci. 85:1185-1192.

Islam, S., M. Abley, N.H. Alam, F. Doussou, A.K.A. Chowdhury, J.F. Desjeux. 2004. Water and Electrolyte Salvage in an Animal Model of Dehydration and Malnutrition. Journal of Pediatric Gastroenterology and Nutrition. 38: 27-33.

Lima, G.H.P. Carvalho, A.A. Figuerido, A.R. Gifoni, A.M. Soares, E.A.T. Silva, R.L. Guerrant. 2002. Effects of an Alanyl-Glutamine-Based Oral Rehydration and Nutrition Therapy Solution on Electrolyte and Water Adsorption in a Rat Model of Secretory Diarrhea Induced by Choera Toxin. Nutrition. 18:458-462.

Noble, R.C. and D. Ogunyemi. 1989. Lipid Changes in the Residual Yolk and Liver of the Chick Immediately after Hatching. Biology of the Neonate. 56:228-236.

Rehrer, N.J. 2001. Fluid and Electrolyte Balance in Ultra-endurance Sport. Sports Medicine. 31:701-715.

Romanoff, A. L. and A. J. Romanoff. 1967. Biochemistry of the Avian Embryo. Wiley and Sons Printing. New York, New York.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed. McGraw-Hill, New York, NY.

Uni, Z., P.R. Ferket, E. Tako, O. Kedar. 2005. In ovo Feeding Improves Energy Status of Late-Term Chick Embryos. Poultry Sci. 84: 764-770.

Voet, D. and J.G. Voet. 2004. Biochemistry, 3rd edition. Wiley and Sons Printing. New York, New York.

Table 5.1a Solutions used in trial 1 including pH.

Trial 1		
Solution	Molarity (mM)	pH
Creatine + KCl	1.0 + 5.5	9.98
CEN + KCL	0.18 + 5.5	8.98
3K Cit+ KCl	5.0 + 5.5	9.33

Table 5.2a Solutions used in trial 2 including pH

Trial 2		
Solution	Molarity (mM)	pH
CEN + KCl + theophylline	0.18 + 5.5 + 1.0	8.12
3kCit + KCl + theophylline	5.0 + 5.5 + 1.0	8.38
Creatine + KCl + theophylline	1 .0 + 5.5 + 1.0	7.85

Table 5.3 Carbohydrate/electrolyte solution (CEN) constitutive elements with mM concentrations as studied in trials 1 and 2.

Solution Strength (mM)	
	CEN [1:40]
Potassium Chloride	0.05
Tripotassium Citrate	2.5×10^{-3}
Magnesium Chloride	3.7×10^{-3}
Zinc Acetate	7.0×10^{-4}
Copper Sulfate	8.7×10^{-6}
Sodium Citrate	2.0×10^{-2}
Sodium Chloride	0.2
Glucose	0.3
Sucrose	0.2

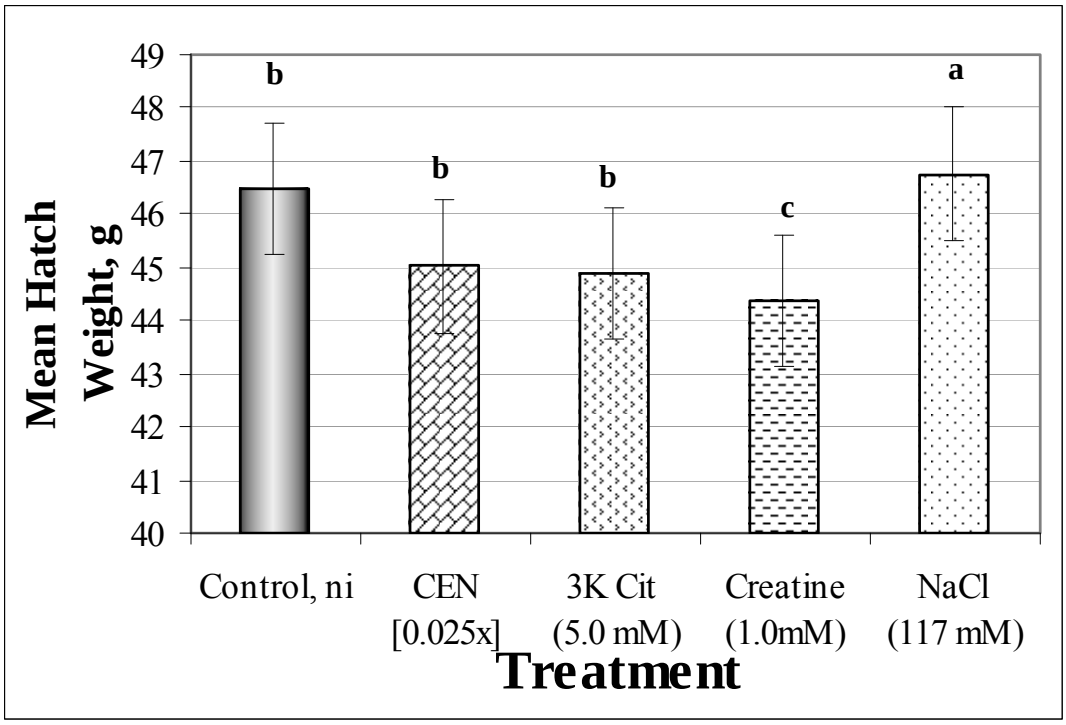

FIGURE 5.1a. Mean hatch weigh (g) of broiler chicks, in the follwong treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), creatine 1.0 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 1[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means

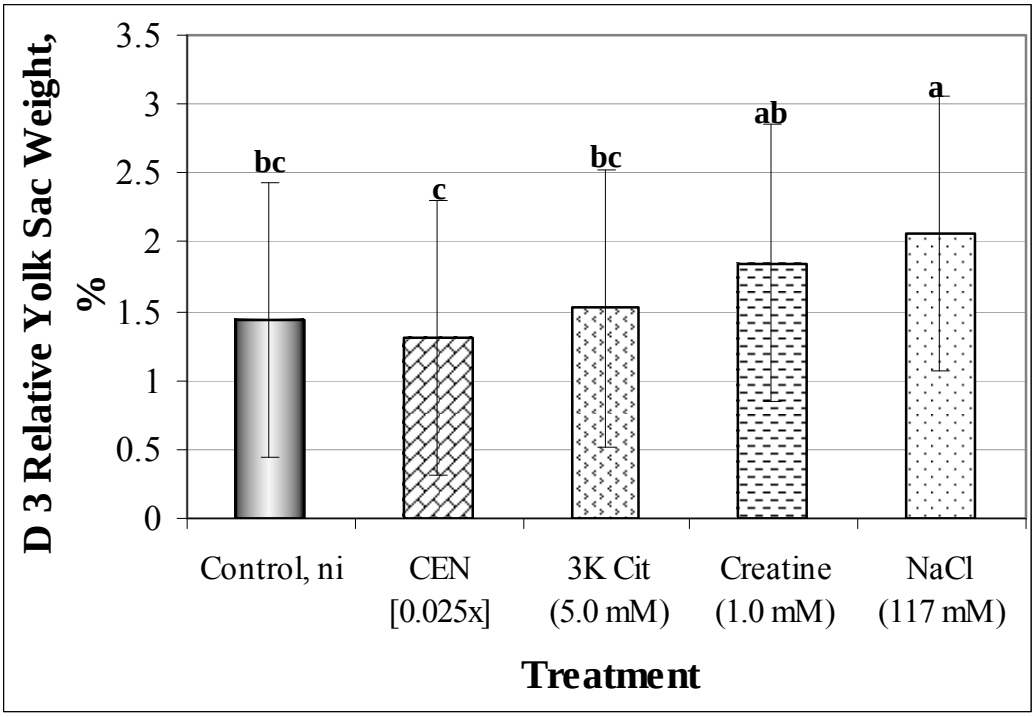

FIGURE 5.2a. Relative yolk sac weights of Day 3 broiler chicks (wet yolk sac weight (g)/ live chick weight (g)), in the following treatment groups, all were carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), creatine 1.0 mM; and a 117 mM saline, and a non-injected control (control, ni) treatment in trial 1[1].

[a-c] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

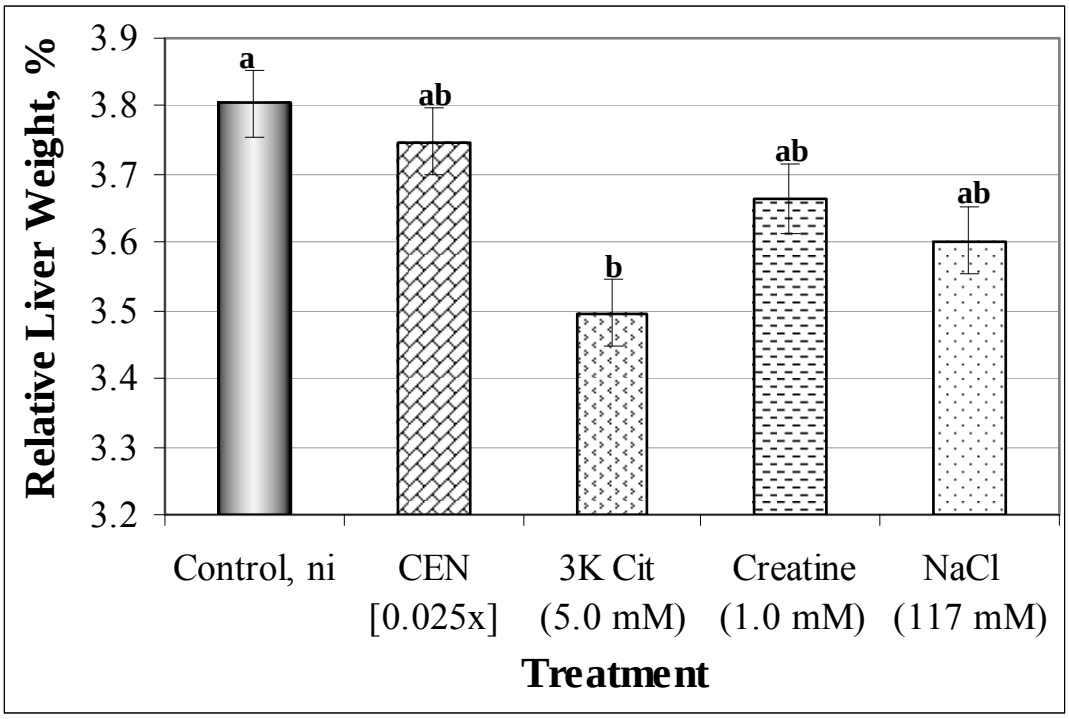

FIGURE 5.3a. Relative liver weight of Day 3 broiler chicks (wet liver weight (g)/ live chick weight(g)), in the following groups carried in 5.5 mM potassium chloride (KCl) and 1.0 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1.0 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[a-b] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

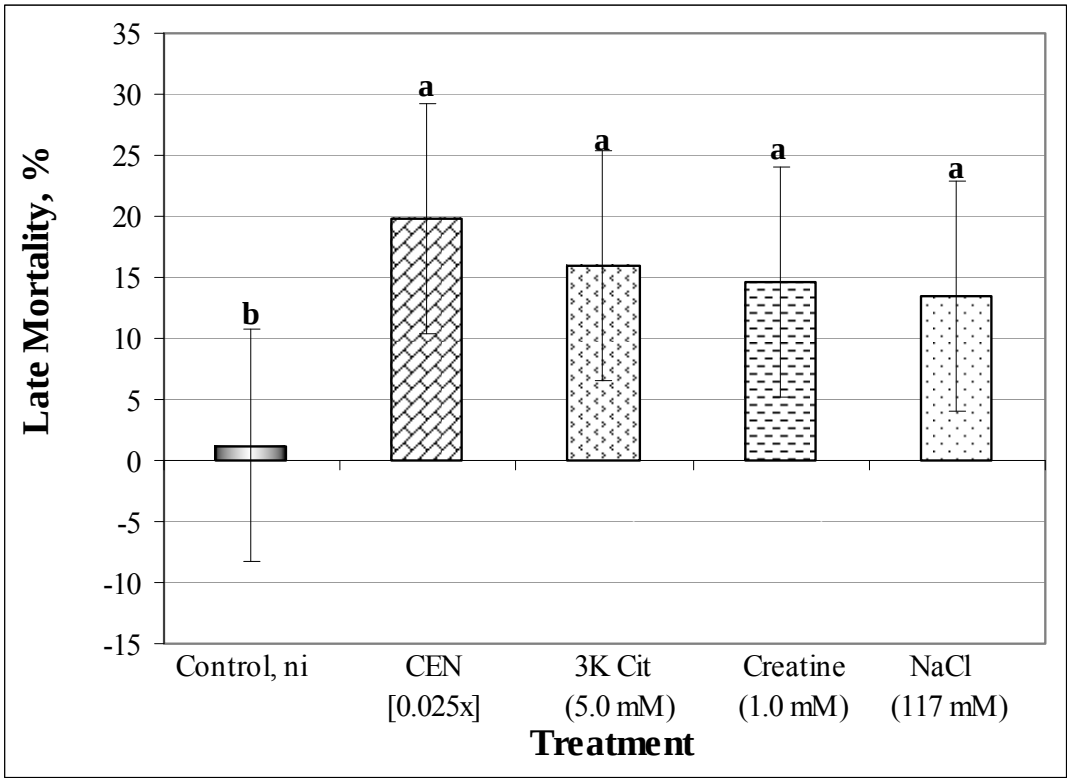

FIGURE 5.4a. Percent late dead (late dead/ total fertile) in the following groups carried in 5.5 mM potassium chloride (KCl) and 1.0 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1.0 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[a-b] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

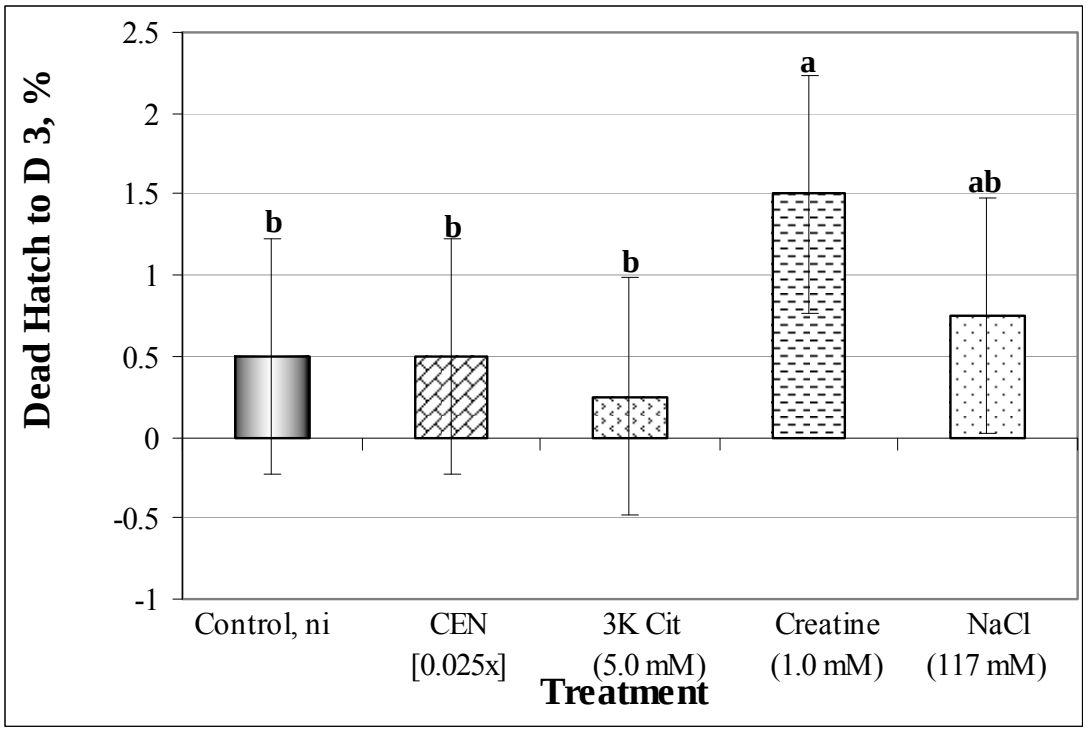

FIGURE 5.5a. Cumulative mortality (hatch to Day 3; Hatch- D 3 dead/ total set) of broiler chicks injected with a in the following groups carried in 5.5 mM potassium chloride (KCl) and 1.0 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1.0 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[a-b] means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n= 4 replicate units used for calculation of means.

CHAPTER VI

SUMMARY

These injections of solutions tested may be able to be administered at Day 18, along with the current vaccine regimen. What was determined was that the electrolyte solutions had no significant effect on embryo mortality. In the individual trials, it was shown that salts have affected relative yolk sac weight. It was also shown that lower concentrations of the electrolyte based solutions had no effect on the embryo. It is only when the electrolyte with a carbohydrate component are injected that an effect on body weight is observed, and this effect is consistent with previous reports of a salt and metabolic solution (Uni *et al.,* 2004).

It is possible that in order to achieve the desired positive effect on body weight that concentrations need to be increased. Addition of the physiological salt, may, aid in maintaining a sufficient hydration status and that this hydration effect is what allows the solution to work more efficiently in the embryo. It was also noticed that when there was a decrease in body weight there was always a corresponding increase in body moisture. This effect is important in that although hatch weight may be decreased, the birds were able to undergo compensatory growth that by Day 3 the weights of birds from treated eggs were not different from those of controls.

However, when used prior to poor brooding in one trial, it was noticed that these injected solutions could be seen as having a negative impact on overall chick livability and growth. Because these conditions occurred at such an early and critical time, it is hard to say definitively that these solutions have any type of impact on growth. It was noticed that when stimulants were added there was no effect on chick hatch weight, but there may have been an increase in weight had it not been for the negative impact of the poor brooding environment in one trial.

With good brooding techniques it is believed that these solutions will promote broiler chick growth rate and increase chick weight. This positive effect on chicks could result in larger meat yield in broilers by processing.

Throughout this study the impact of the base solute has been shown. Many researchers use a base salt, typically sodium chloride, for their injectible solutions without regard to the osmotic effect that the sodium chloride could have on the experiment. It has been shown, through comparative analysis, that both sodium chloride and potassium chloride can have lesser or greater effects on embryos and post hatch chicks when used in conjunction with addition solutes. These base solutions should be stringently analyzed and rigorously quantified in terms of individual effects before attempts are made to use these salts as a “harmless” carrier ion solution for substances whose effects are of main interest to investigators.

APPENDIX A

CALCULATED mOsm FOR SELECT SOLUTIONS IN CHAPTERS III, IV, AND V

TABLE 3.1b Calculated mOsm for all solutions tested in trial 1, where miliosmolarities were calculated using the formula mOsm= Φ n C, where Φ was set equal to 0.95 dissacoiation, n is the number of dissociated ions, and C is the concentration.

Solution	Conc	N	mOsm
Sodium Acetate	110	2	209
Sodium Acetate	118	2	224.2
Sodium Acetate	125	2	237.5
Sodium Acetate	130	2	247
Sodium Phosphate	0.5	3	1.425
Sodium Phosphate	1	3	2.85
Sodium Phosphate	1.5	3	4.275
Sodium Phosphate	2	3	5.7
Potassium Chloride	4	2	7.6
Potassium Chloride	4.5	2	8.55
Potassium Chloride	5	2	9.5
Potassium Chloride	5.5	2	10.45
Sodium Chloride	117	2	222.3

TABLE 3.4b Calculated mOsm for all solutions tested in trial 4, where miliosmolarities were calculated using the formula mOsm= Φ n C, where Φ was set equal to 0.95 dissacoiation, n is the number of dissociated ions, and C is the concentration.

Solution	Conc	n	Calculated mOsm
Caffiene	1	1	0.95
Theophylline	1	1	0.95
Creatine	1	1	0.95
Phosphocrreatine	1	1	0.95
Sodium Chloride	117	2	222.3

TABLE 4.1b Calculated mOsm for all solutions tested in trial 1 (Chapter IV), where miliosmolarities were calculated using the formula mOsm= Φ n C, where Φ was set equal to 00.95 dissacoiation, n is the number of dissociated ions, and C is the concentration.

Solution in potassium chloride(5.5 mM)	mOsm
Sodium Phosphate (1.0 mM)	13.3
Tripotassium citrate (5.0 mM)	29.45
Creatine(1.0 mM)	11.4
CEN	11.36673
Sodium Chloride (117 mM)	222.3

TABLE 5.1b Calculated mOsm for all solutions tested in trial 1 (Chapter IV), where miliosmolarities were calculated using the formula mOsm= Φ n C, where Φ was set equal to 00.95 dissacoiation, n is the number of dissociated ions, and C is the concentration.

Solution in potassium chloride(5.5 mM)	mOsm
Sodium Phosphate (1.0 mM)	13.3
Tripotassium citrate (5.0 mM)	29.45
Creatine(1.0 mM)	11.4
CEN	11.36673
Sodium Chloride (117 mM)	222.3

TABLE 5.2b Calculated mOsm for all solutions tested in trial 1, where miliosmolarities were calculated using the formula mOsm= Φ n C, where Φ was set equal to 0.95 dissacoiation, n is the number of dissociated ions, and C is the concentration.

Solution in KCl (5.5 mM) +Theophylline (1.0 mM)	mOsm
CEN	12.31673
Tripotassium Citrate (5.0 mM)	30.4
Creatine(1.0 mM)	12.35
Sodium Chloride (117 mM)	222.3

APPENDIX B

SIGNIFICANT DATA NORMALIZED FOR mOsm IN CHAPTER III

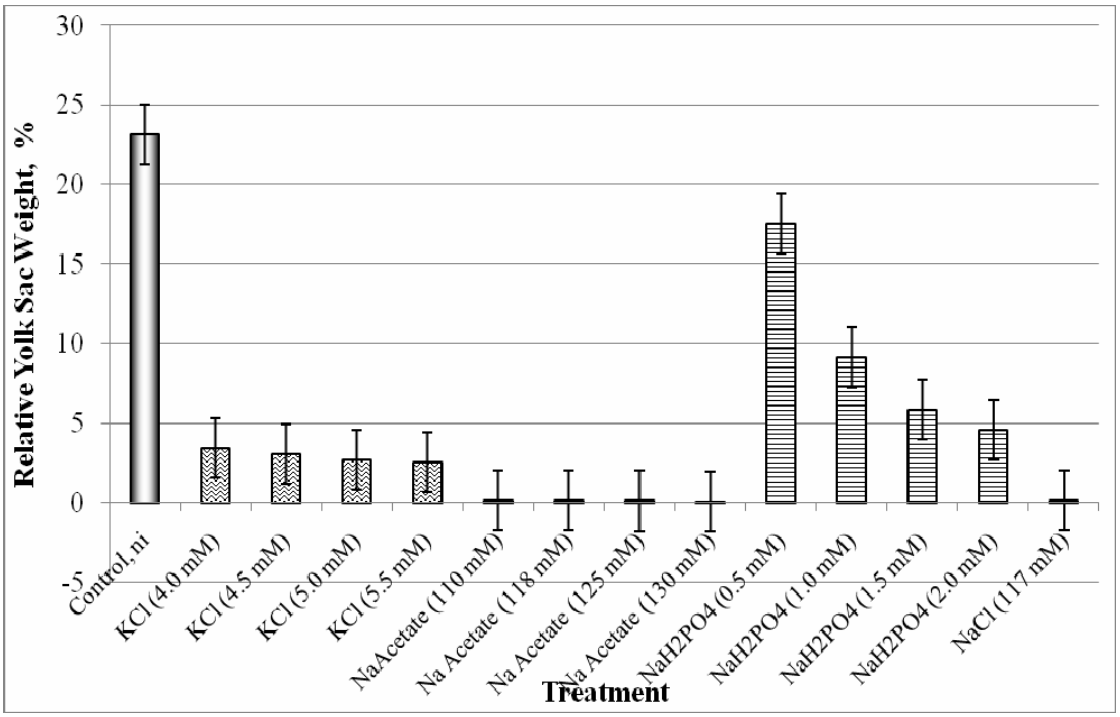

FIGURE 3.1b. Relative yolk sac weight of Day 18 broiler embryos ((wet yolk sac- dry yolk sac)/ wet yolk sac) normalized by mOsm in the following treatment groups: potassium chloride (KCl) at 4.0, 4.5, 5.0, and 5.5 mM; sodium acetate (Na Acetate) at 110, 118, 125, and 130 mM; sodium dihydrogenphosphate (NaH_2PO_4) at 0.5, 1.0, 1.5, and 2.0 mM; saline at 117 mM, and a non-injected control (control, ni) in trial 1.[1]

[1]n= 4 replicate units used for calculation of means.

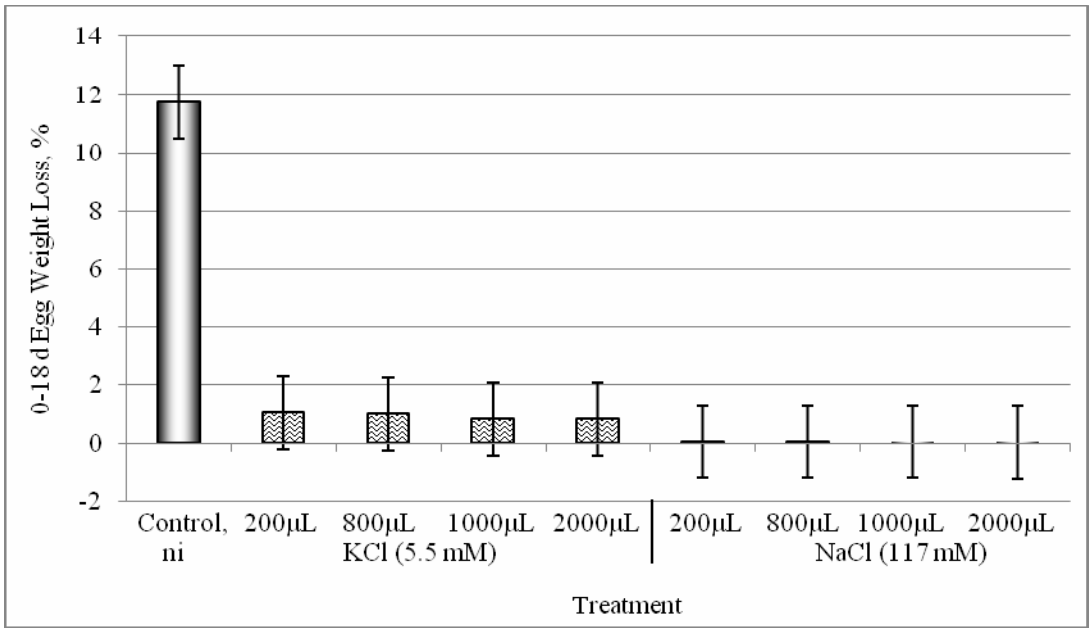

FIGURE 3.2b. Percent 0- 18 d incubational egg weight loss (0-18 d water loss (g)/ set egg weight (g)), normalized for calculated mOsm, in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control, ni) in trial 3[1].

[1]n= 2 replicate units used for calculation of means.

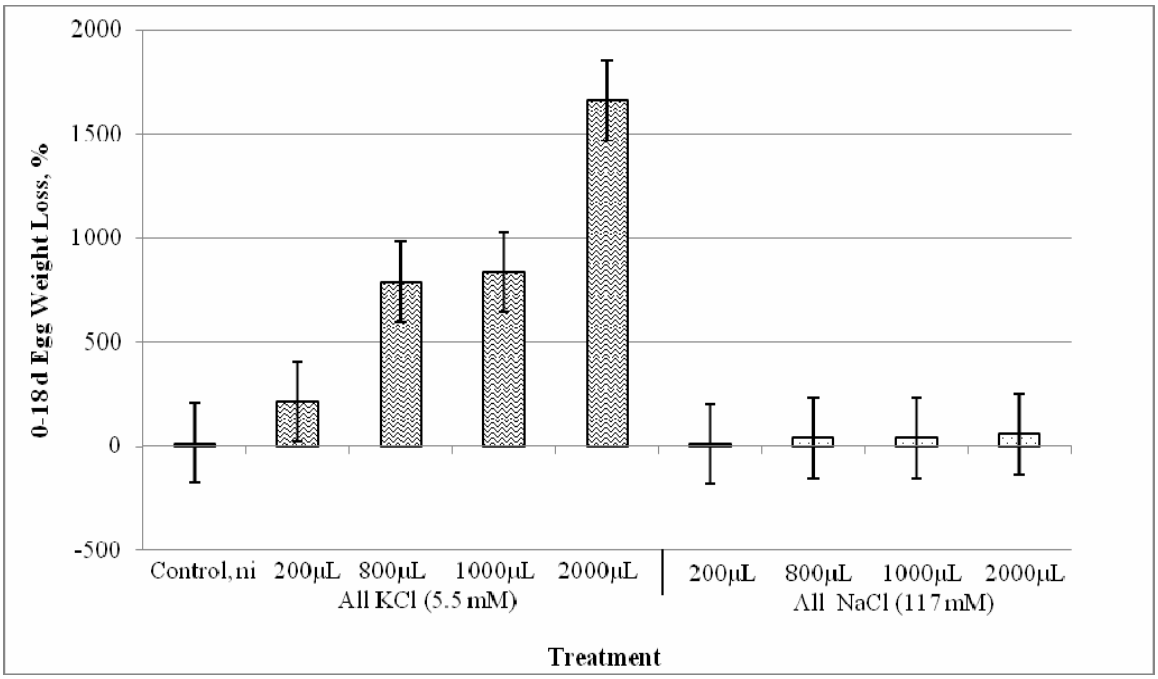

FIGURE 3.2c. Percent 0- 18 d incubational egg weight loss (0-18 d water loss (g)/ set egg weight (g)), normalized for calculated mOsm and volume injected, in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control, ni) in trial 3[1].

[1]n= 2 replicate units used for calculation of means.

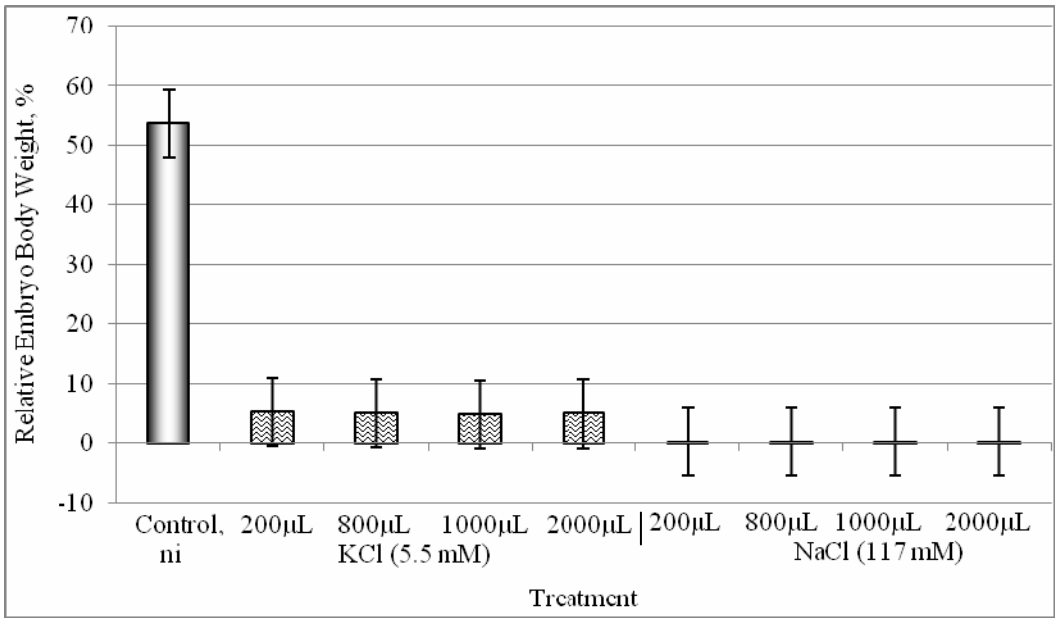

FIGURE 3.3b. Relative embryo body weights of Day18 broiler embryos (wet embryo (g)/dry embryo (g)), normalized for mOsm, in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control) in trial 3[1].

[1]n= 2 replicate units used for calculation of means.

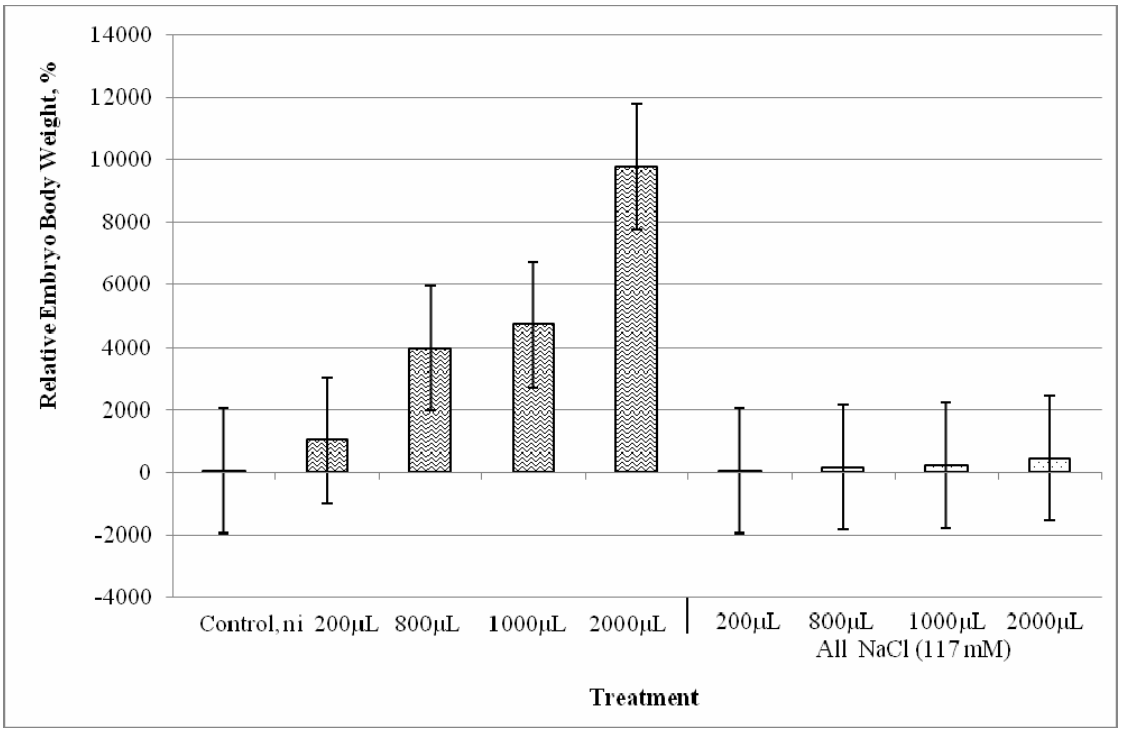

FIGURE 3.3c. Relative embryo body weights of Day18 broiler embryos (wet embryo (g)/dry embryo (g)), normalized for mOsm and volume, in the following treatment groups: 5.5 mM potassium chloride (KCl) at 200, 800, 1000, and 2000 µL volumes; 117 mM saline at 200, 800, 1000, and 2000 µL volumes; and a non-injected control (control) in trial 3[1].

[1]n= 2 replicate units used for calculation of means.

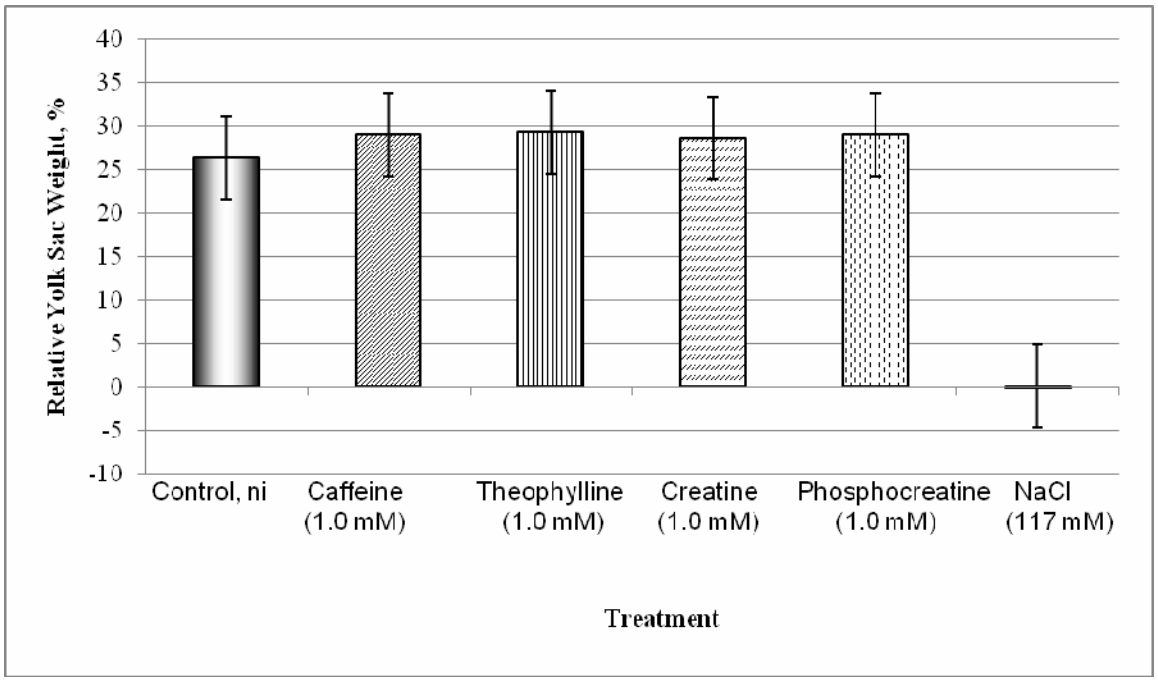

FIGURE 3.4b. Relative yolk sac weight of Day 18 broiler embryos (wet yolk sac (g)/ D 18 egg weight (g)), normalized for mOsm, in the following treatment groups: 1 mM caffeine, theophylline, creatine and phosphocreatine; 117 mM saline; and a non-injected control (control, ni) in trial 4[1].

[1]n= 2 replicate units used for calculation of means.

APPENDIX C

SIGNIFICANT DATA NORMALIZED FOR mOsm IN CHAPTER IV

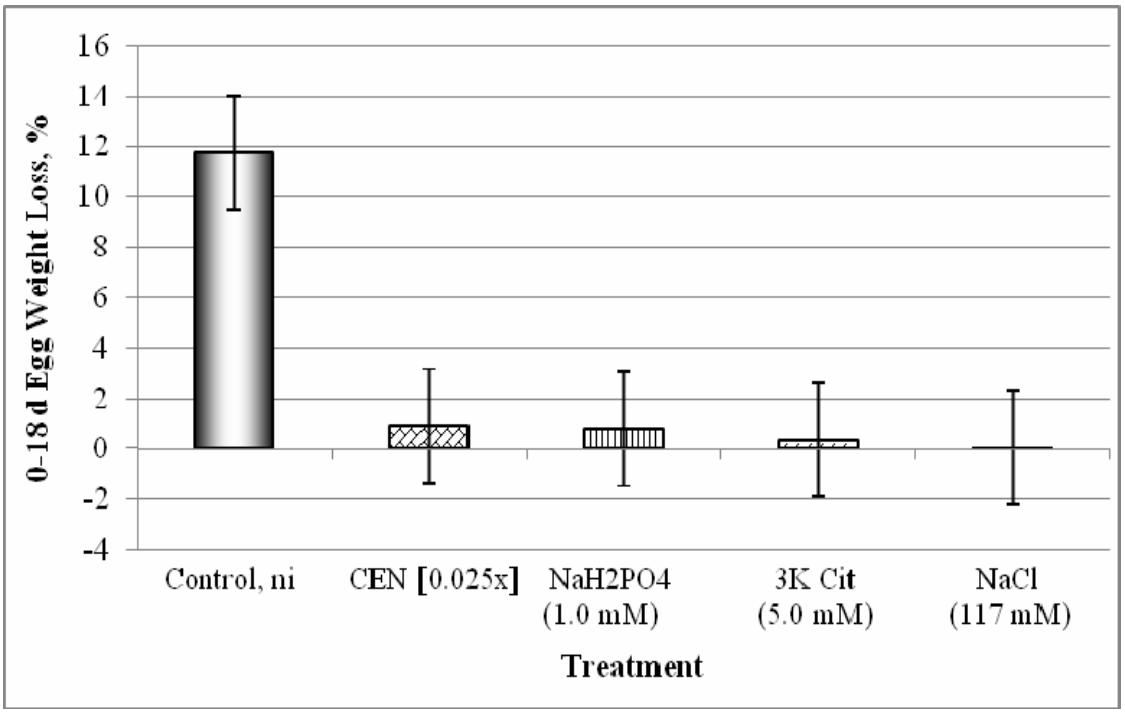

FIGURE 4.1b. Percent 0-18 d incubational egg weight loss (0-18 d Δ (g)/0 d (g)), normalized for mOsm, in the following treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and non-injected control (control, ni) treatments in trial 1[1].

[1]n= 4 replicate units used for calculation of means.

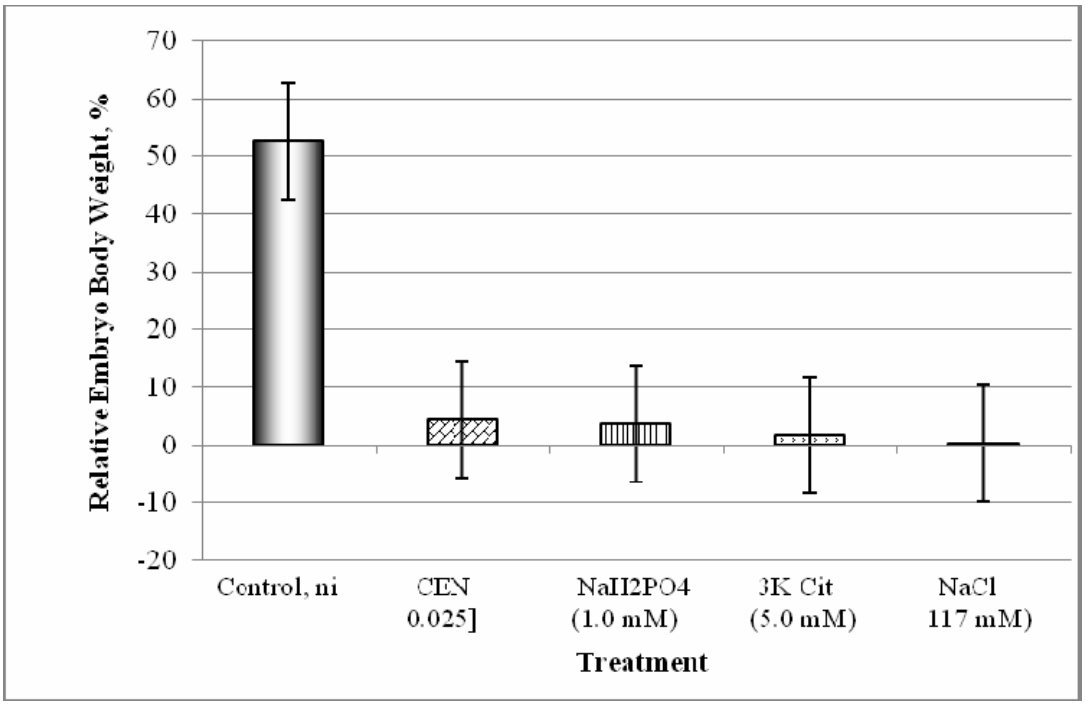

FIGURE 4.2b. Relative embryo body weight of Day 18 broiler embryos (wet embryo (g)/ D 18 egg weight (g)), normalized for mOsm, in the following treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and non-injected control (control, ni) treatments in trial 1[1].

[1]n= 4 replicate units used for calculation of means.

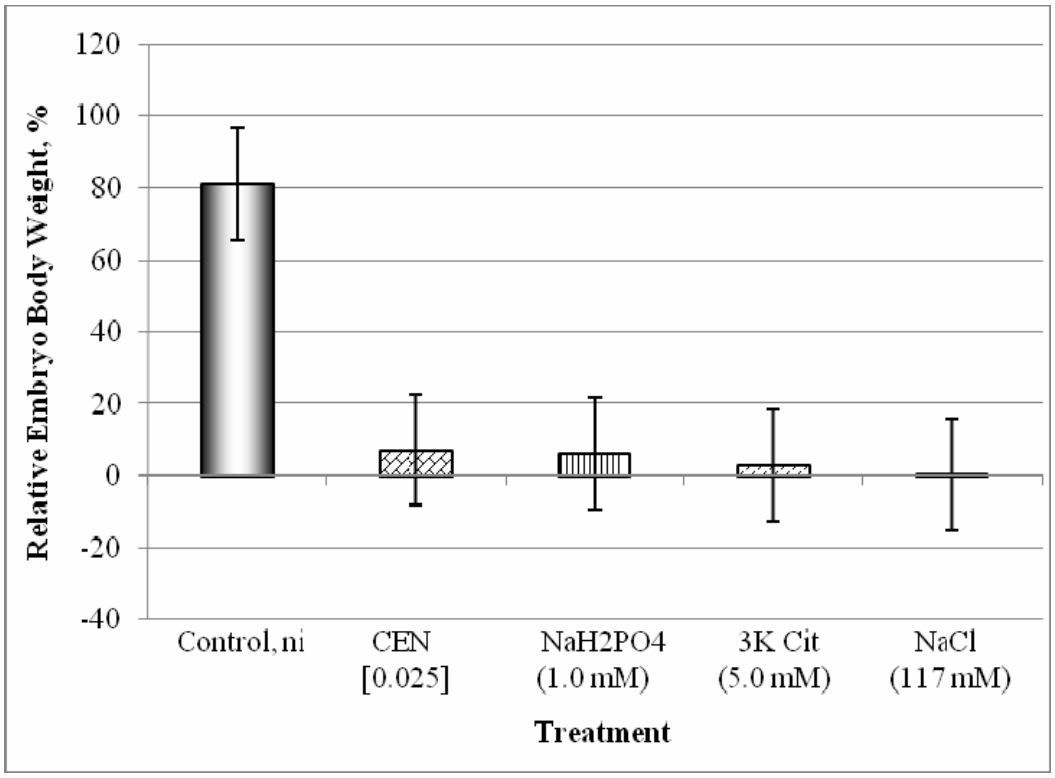

FIGURE 4.3b. Percent embryo body moisture of Day 18 broiler embryos ((wet embry-dry embryo) (g)/ wet embryo(g)), normalized for mOsm, in the following treatment groups, where treatments were all carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), and sodium dihydrogenphosphate (NaH_2PO_4); and 117 mM saline, and a non-injected control (control, ni) treatments in trial 1[1].

[1]n= 4 replicate units used for calculation of means.

APPENDIX D

SIGNIFICANT DATA NORMALIZED FOR mOsm IN CHAPTER V

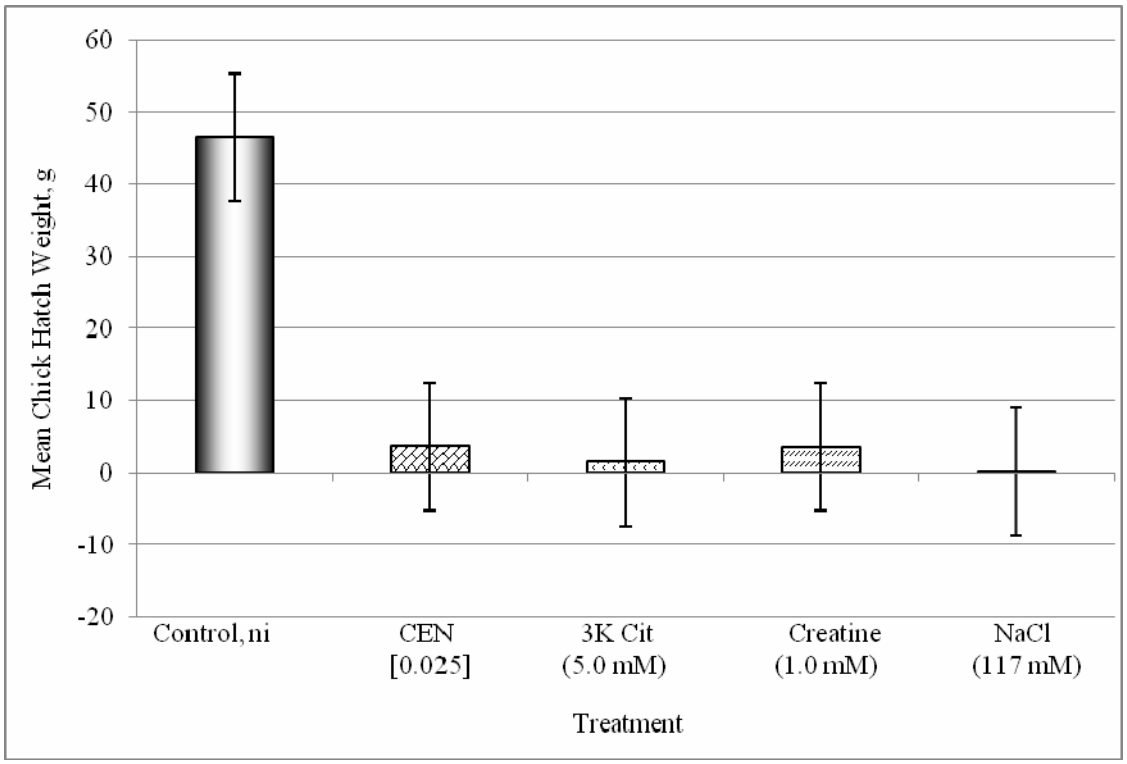

FIGURE 5.1b. Mean hatch weigh (g) of broiler chicks, normalized for mOsm, in the following treatment groups carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), creatine 1 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 1[1].

[1]n= 4 replicate units used for calculation of means

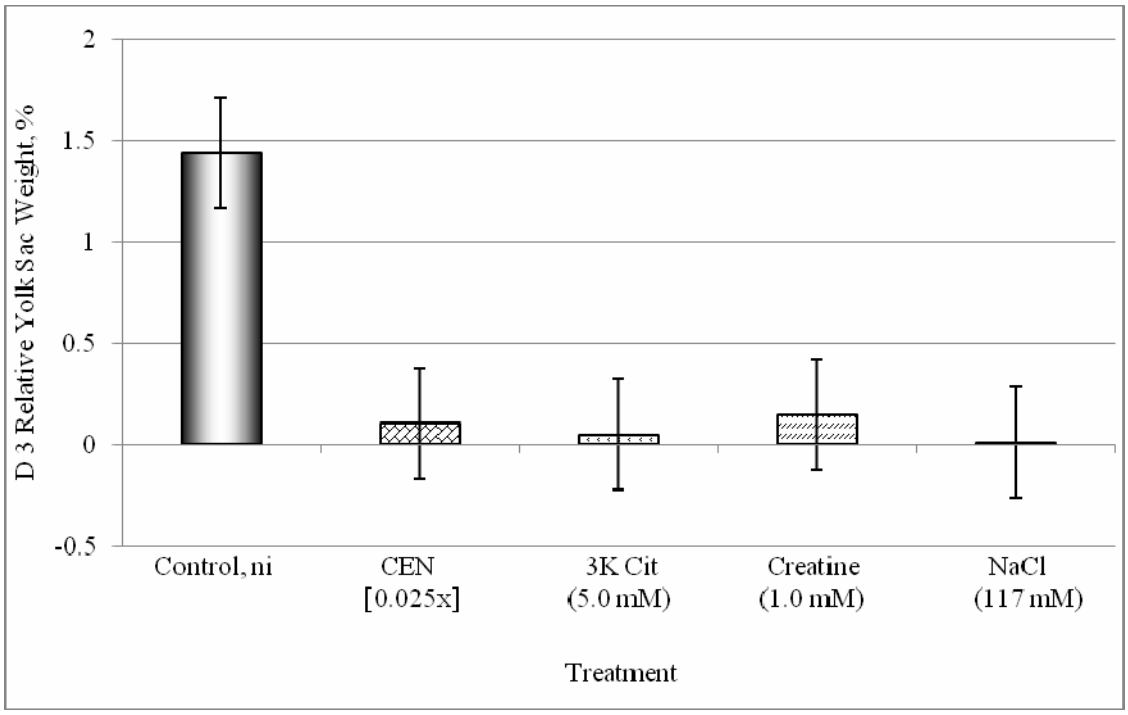

FIGURE 5.2b. Relative yolk sac weights of Day 3 broiler chicks (wet yolk sac weight (g)/ live chick weight (g)), normalized for mOsm, in the following treatment groups, all were carried in 5.5 mM potassium chloride (KCl): tripostassium citrate (3K Cit), a carbohydrate/ electrolyte solution (CEN), creatine 1 mM; and a 117 mM saline, and a non-injected control (control, ni) treatment in trial 1[1].

[1]n= 4 replicate units used for calculation of means.

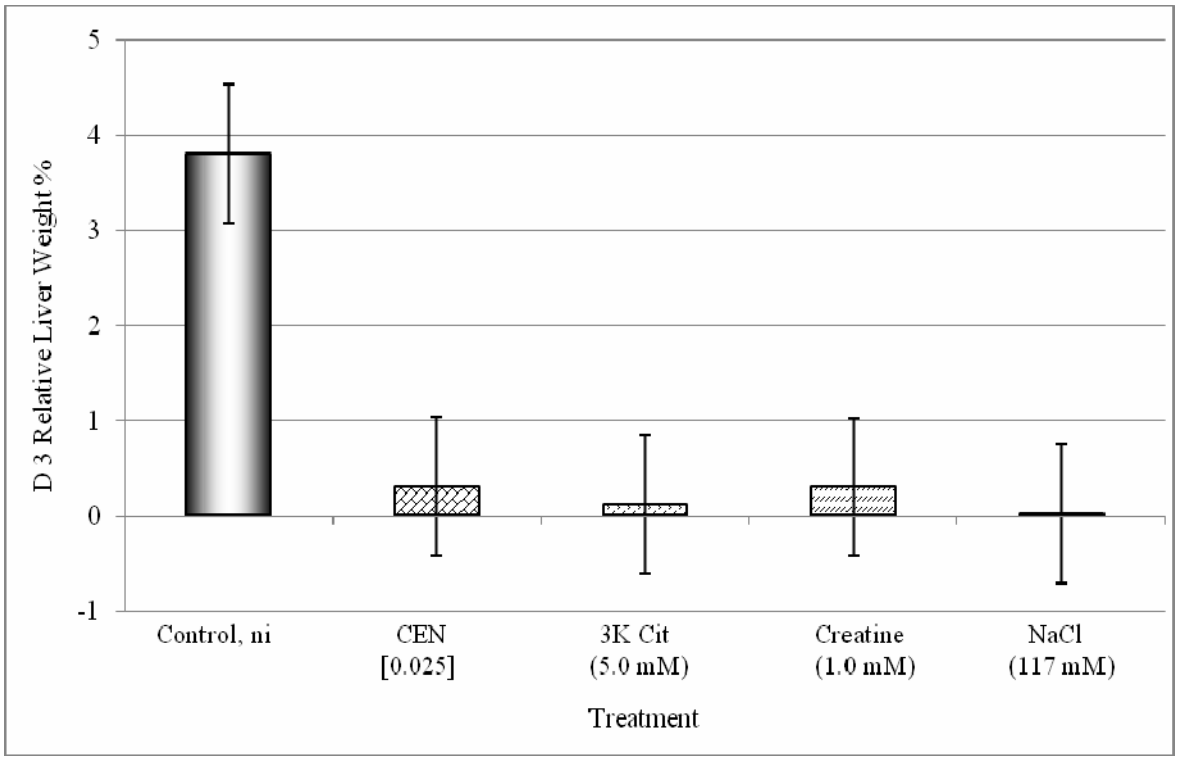

FIGURE 5.3b. Relative liver weight of Day 3 broiler chicks (wet liver weight (g)/ live chick weight(g)), normalized for mOsm, in the following groups carried in 5.5 mM potassium chloride (KCl) and 1 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[1]n= 4 replicate units used for calculation of means.

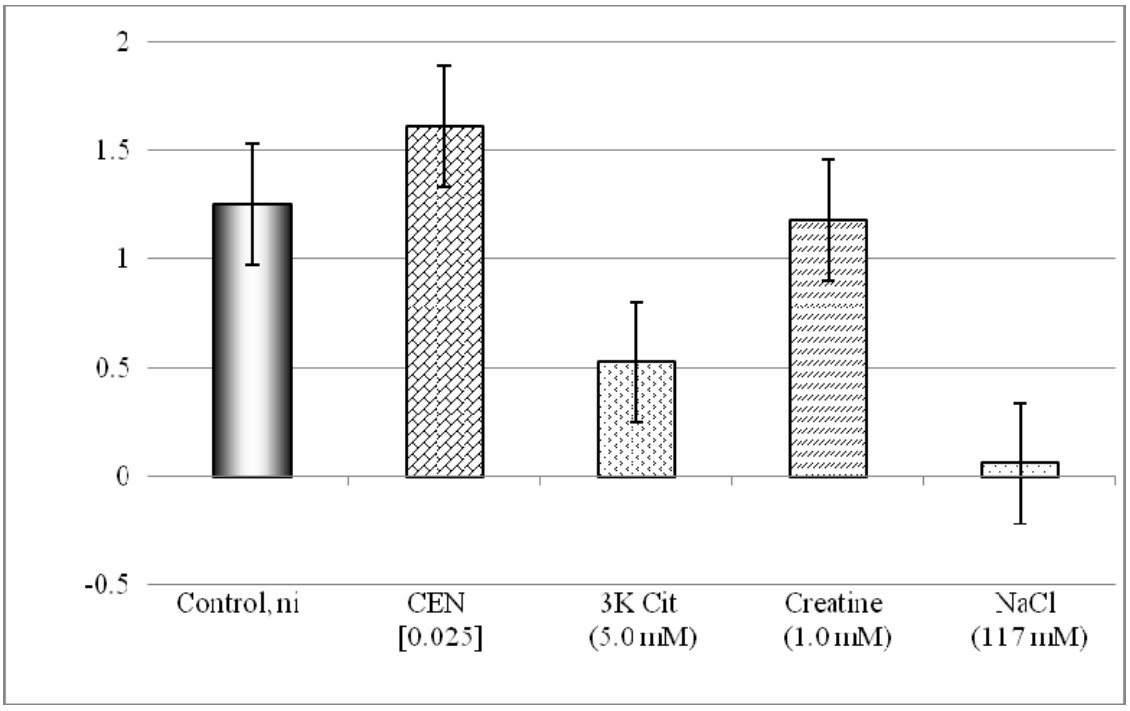

FIGURE 5.4b. Percent late dead (late dead/ total fertile), normalized for mOsm, in the following groups carried in 5.5 mM potassium chloride (KCl) and 1 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[1]n= 4 replicate units used for calculation of means.

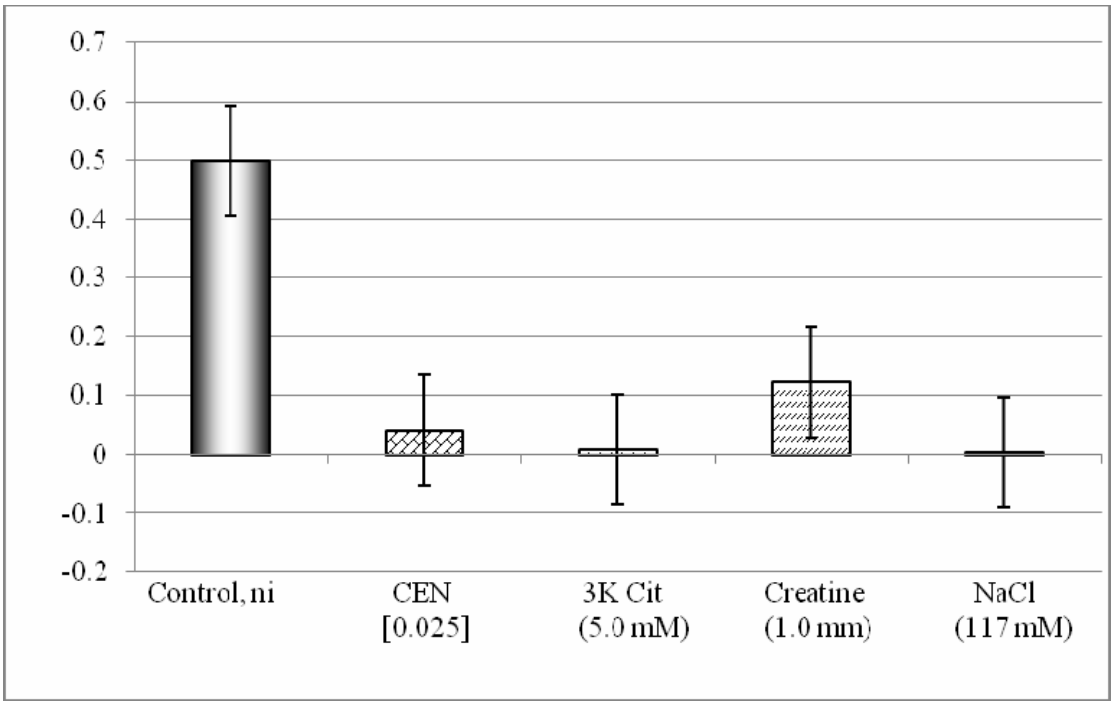

FIGURE 5.5b. Cumulative mortality (hatch to Day 3; Hatch- D 3 dead/ total set), normalized for mOsm, of broiler chicks injected with a in the following groups carried in 5.5 mM potassium chloride (KCl) and 1 mM theophylline: tripostassium citrate (3K Cit), a carbohydrate electrolyte solution (CEN), creatine 1 mM; also a 117 mM saline, and a non-injected control (control, ni) treatment in trial 2[1].

[1]n= 4 replicate units used for calculation of means.

APPENDIX E

CROSS COMPARISON OF STIMULANTS CARRIED IN WATER VERSUS 5.5 mM POTASSIUM CHLORIDE

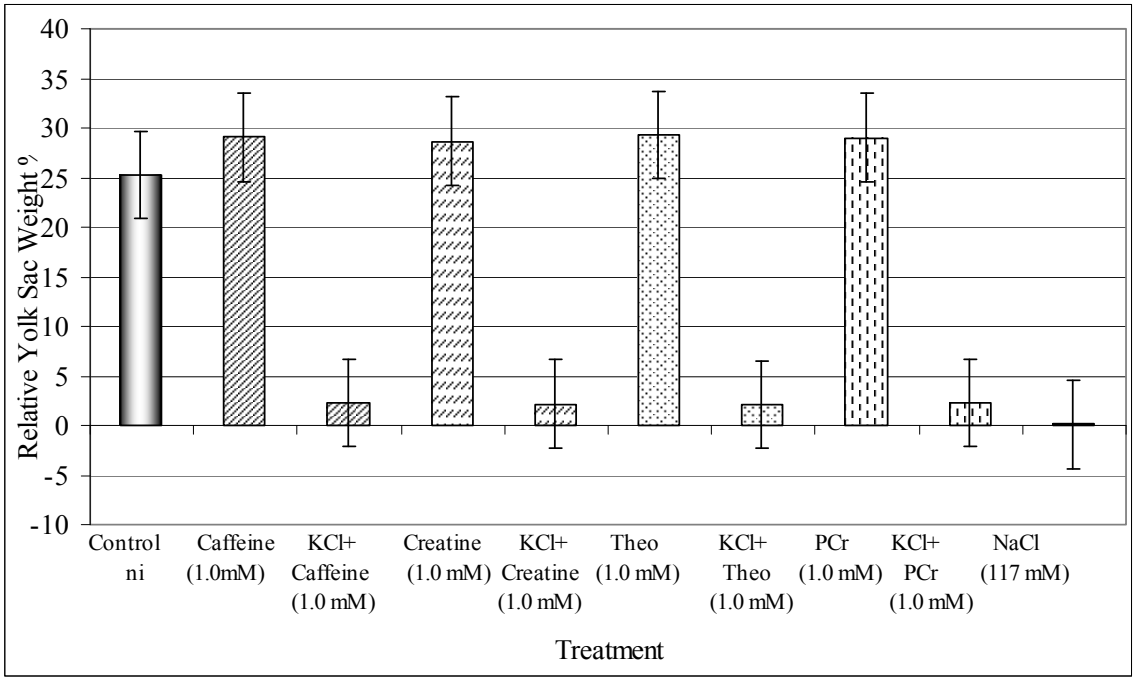

FIGURE 4.4. Effects of stimulants on relative yolk sac weight when carried in water versus in 5.5 mM potassium chloride (KCl +) and when compare to a control non-injected (control, ni) and 117 mM NaCl as evaluated by the non-statistical comparison across two separate trials (trial 4, Chapter III and trial 2, Chapter IV).