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Eggshell Conductance of Turkey Eggs Affects Cardiac Physiology and Subsequent Embryo Survival[1]

V.L. Christensen[2,3], L.G. Bagley[4], T. Olson[4], J.L. Grimes[3] and D.T. Ort[3]
[3]Department of Poultry Science, College of Agriculture and Life Sciences,
North Carolina State University, Raleigh, North Carolina 27695-7608, USA
[4]Moroni Turkey Hatchery, Moroni, UT 84646, USA

Abstract: Embryo heart rates were measured on 400 fertilized turkey eggs (399 viable embryos) at 4 day intervals beginning at day 12 of development. Heart rates varied directly with eggshell porosity and were significantly and positively correlated with eggshell conductance (G) and conductance constants (k) but not with initial egg weight. When only eggs with embryos that died were analyzed the significant correlation coefficients increased. In a second experiment, eggshell pores were occluded to reduce G then heart rates were measured. Heart rates decreased concomitantly with decreases in G. In the final experiment, approximately 15,912 eggs were weighed individually to calculate G for each egg and were then incubated. Embryo survival was noted in High and Low G groups. Embryo heart rate and cardiac physiology in each group was measured. Low G reduced heart rates and improved embryo survival and cardiac physiology compared to High G. Thus, cardiomyopathy due to High G and its consequent lack of energy for myocardial function may contribute to turkey embryo mortality late in development.

Key words: Eggshell conductance, embryo survival, cardiac physiology

Introduction
When a portion of the avian eggshell is covered with impermeable plastic membranes, growth is retarded (Metcalfe *et al.*, 1981; McCutcheon *et al.*, 1982; Bagley and Christensen, 1989). (McCutcheon *et al.*, 1982) also observed a significant reduction in chick embryonic liver and heart weights when the eggshells were covered. Additionally, (Smith *et al.*, 1969) showed that the overall growth of chick embryos can be retarded by incubating in hypoxia at high altitude. Stock and Metcalfe (1984) suggested that depressed metabolism from low oxygen pressure limited organ growth and maturation; thus the maturation of the turkey embryo may be limited normally by the availability of oxygen. Thicker shells (Christensen *et al.*, 2006a) and increased eggshell conductance (Christensen *et al.*, 2006b) have recently been shown to be factors in embryo heart development, but the results were confounded with a dietary calcium supplement used to create thicker shells because ionic calcium may affect cardiac function. Eggshell conductance (G) is a component of a multifactor relationship involving egg weight and the length of the incubation period that is called the conductance constant (k). The k varies directly with G and the incubation period but inversely with egg weight. We hypothesize that the relationship among egg weight, G and length of the incubation period affect heart development and the eventual survival of turkey embryos.

Materials and Methods
Correlation experiment: The initial study correlated heart rates of embryos with egg characteristics of weight, G and k. Four hundred randomly selected eggs were obtained that were produced by flocks of Large White turkeys. The eggs were numbered, weighed and then set in an incubator. During incubation they were exposed to uniform conditions (37.5°C and 50% RH) until the end of the 24th day of development. At the beginning of the 25th day of development, the eggs were weighed a second time to determine the G of each egg using the calibrated egg technique of (Tullett, 1981). Heart rates for individual embryos were recorded at 4 day intervals beginning at day 12 (Christensen *et al.*, 2006a,b). Heart rates, egg weight, G, and k were correlated using (SAS, 1998).

Reduced G experiment: Correlation analysis gives limited information about cause and effect but only the degree of association of the factors. In the second study we reduced G artificially to test its effects on turkey embryo heart rates. G was reduced with paraffin then heart rates were measured at 4 day intervals as described previously. Approximately 300 fertilized turkey eggs were divided randomly into three groups of 100 eggs each. The first group was covered partially by dipping the narrow end of the egg into paraffin to a depth of 2 cm. A second group was treated similarly but was dipped only to a depth of 1 cm. The third group was not treated and served as the controls.

Following treatment, the eggs were weighed and placed into the incubator then incubated as described in Experiment 1. The G of each group was then calculated (Tullett, 1981). Mean heart rates of each G reduction

treatment were then compared using the general linear models procedure and least square means separation test (SAS, 1998).

G, heart rate and embryo survival experiment: The effects of G on embryo cardiac energy metabolism and survival were examined in Experiment 3. In each of three replicate trials, approximately 5,304 eggs were numbered and weighed (nearest 0.01 g) prior to setting in incubators. All eggs were weighed again at the 24th day of development to determine G by the calibrated egg technique (Tullett, 1981). The eggs were sorted into High and Low G groups at that time and placed into hatching trays. Each tray contained 136 eggs. The trays were arranged in racks as alternating rows of High and Low G eggs on each of three racks. Each rack accommodated 13 trays. The trays (39 total trays per trial) served as the experimental unit for the embryo survival analysis. Embryo survival was recorded as the percentage of hatched embryos counted in each tray at the completion of 28 days of incubation. Eggs on each tray that did not hatch were broken open, examined for embryo development and categorized by approximate developmental age at death. Percentages of embryo survival and death for each G treatment were calculated, transformed by arcsine transformations then analyzed using the general linear models procedure of (SAS, 1998).

Time of hatching was observed on 13 trays within each trial by counting the number of poults hatched at 6 hour intervals beginning at 630 hours of development. The rate at which embryos advanced from one stage of development to another was also measured. Embryos were staged (Hamburger and Oppenheim, 1970; Christensen *et al.*, 2006b) at the same times when hatched poults were counted by using a candling light. The percentage of total poults hatched at each time was the variable analyzed for time of hatching by the general linear models procedure of (SAS, 1998). The number of hours required to attain a given stage as well as the number of hours the embryo remained at that stage prior to advancing to the next stage were the variables.

Tissues were sampled at days 27 and 28 of development by procedures described by (Christensen *et al.*, 2006ab). Heart rates were measured at days 25, 26 and 27 of development using 15 embryos per trial per treatment (total of 45 per treatment). Five embryos or poults per treatment per trial (a total of 15 per treatment) were weighed (nearest 0.01 g) at pipping (day 27) and hatching (day 28) with and without yolk. Blood samples were collected following decapitation then hearts and livers were quickly dissected, weighed and frozen (-22°C). Blood was centrifuged (700 x g) for 10 minutes then the plasma was recovered and frozen (-22°C). Subsequently, cardiac and hepatic tissues were homogenized in 7% cold perchloric acid and analyzed

for glycogen and lactate concentrations (Christensen *et al.*, 2006ab). The blood plasma was analyzed for glucose and lactate concentrations as well as for CK and LDH activities. Tissue data were analyzed by the general linear models procedure of (SAS, 1998) with individual bird as the experimental unit. Means differing significantly were separated with the least square procedure. Probability was based on P<0.05.

Results

Correlation experiment: Significant positive correlations between G, k and heart rates were seen at days 20 and 24 of embryo development (Table 1). Egg weights were not correlated significantly with heart rates. The correlation coefficients were small in magnitude when all eggs were included in the analysis, and based on the square of the correlation coefficient only 2 to 3% of the variation could be accounted for by G or k. However, when only the eggs containing dead embryos were analyzed, coefficients doubled in magnitude and more than 10% of the variation could be explained by G or k.

Reduced G experiment: The positive correlation between G and heart rates led to a subsequent trial to demonstrate directly that low G reduces heart rates. The G of randomly selected eggs was reduced artificially and heart rates were measured. Lower G decreased heart rates of turkey embryos in a stepwise fashion (Table 2). The data verify that reduced G also slows heart rates and confirms results of the positive correlation between G and heart rates seen in the initial trial.

G, heart rate and embryo survival experiment: No significant differences were seen in the average weights of eggs in Low and High groups (High = 85.8g, Low = 85.9g, Mean±SEM = 85.8 ± 0.1), but the G and k values of each selected group differed significantly (High G = 18.9 mg H_2O/d/mmHg, Low G = 18.4 mg H_2O/d/mmHg, Overall mean + SEM = 18.6 ± 0.1; High k = 6.35, Low k = 6.25, Overall mean±SEM = 6.29 ± 0.01). Heart rates of embryos in High G eggs were significantly faster than those in Low at days 25, 26 and 27 of development (Table 3). More (7%) embryos in Low G eggs survived than in High G (Table 4). Nearly 5% more deaths near the plateau stage were in High than Low G eggs. Nearly 1.3% more embryos from High G eggs than in Low were able to emerge from the shell following external pipping. Embryo weights did not differ at day 27 between the two G groups, but at hatching Low G poults weighed more than High (Table 5). Hearts (mg) weighed more in Low than High G eggs at both stages of development (Table 6). In contrast to the heart, the weight of the liver was reduced in Low G eggs compared to High (Table 7). Elevated myocardial glycogen and depressed lactate concentrations were seen in Low G embryos compared to High (Table 8). It follows then that the ratio of total

Table 1: Correlation of turkey embryo heart rates with egg weight and eggshell conductance (G) and conductance constants (k)

Variable	Para-meter	Day of development			
		12	16	20	24
-		---------- All eggs (n = 399) ----------			
Egg weight	r^2	0.01	0.01	0.01	0.07
	P	NS	NS	NS	NS
G	r^2	0.09	0.09	0.15	0.12
	P	0.08	0.09	0.01	0.03
k	r^2	0.09	0.09	0.14	0.09
	P	0.08	0.08	0.008	0.09
		---- Eggs that did not hatch (n = 63) ---			
Egg weight	r^2	-0.01	0.02	0.02	0.11
	P	NS	NS	NS	NS
G	r^2	0.14	0.01	0.32	0.08
	P	NS	NS	0.01	NS
k	r2	0.13	-0.01	0.34	0.05
	P	NS	NS	0.006	NS

Table 2: Effect of reducing eggshell conductance on heart rates (bpm) of turkey embryos

Treatment	Day of development			
	12	16	20	24
Control	248[a]	227[a]	225[a]	231[a]
1 cm	223[b]	216[b]	202[b]	221[b]
2 cm	210[c]	197[c]	201[b]	213[c]
Mean±SEM	228±1	218±1	212±1	224±1
Probability	0.0001	0.0001	0.0001	0.0001

Table 3: Heart rates (bpm) of turkey embryos developing in eggs with High and Low eggshell conductance (G)

G	Day of development		
	25	26	27
High	232[a]	230[a]	244[a]
Low	221[b]	220[b]	227[b]
Mean±SEM	226±1	225±1	236±1
Probability	0.0001	0.0001	0.0001

Table 4: Embryo survival rates and times of embryo death (% of fertile eggs) of turkey embryos developing in eggs with High and Low conductance (G)

G	Measurement			
	Embryo survival (%)	Mortality at wk 1 (%)	Mortality at wk 4 (%)	Mortality at pipping (%)
High	80.7[b]	4.0	9.9[a]	4.1[a]
Low	87.0[a]	4.4	4.4[b]	2.8[b]
Mean±SEM	84.2±0.4	4.2±0.2	6.8±0.3	3.4±0.2
Probability	0.0001	NS	0.0001	0.0001

glycogen to total lactate was greater in the myocardium of Low G embryos than High. Hepatic tissue from Low G embryos had elevated lactate concentrations at d 27 of development but a greater glycogen to lactate ratio than did High (Table 9). At d 28 no differences were noted in liver glycogen or lactate.

Plasma CK activities did not differ among embryos from

Table 5: Body weight (g) with and without yolk of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	------------------ With ------------------	
High	63.3	59.3[b]
Low	64.6	61.7[a]
Mean±SEM	63.9±0.7	60.5±0.7
Probability	NS	0.10
	---------------- Without --------------	
High	51.0	49.1[b]
Low	51.7	51.7[a]
Mean±SEM	51.4±0.5	50.4±0.5
Probability	NS	0.009
	-------------- Yolk weight ------------	
High	12.3	10.2
Low	12.9	9.9
Mean±SEM	12.6±0.4	10.1±0.4
Probability	NS	NS

Table 6: Heart weight (g) of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	---------- Absolute (mg) ----------	
High	271[b]	314[b]
Low	288[a]	346[a]
Mean±SEM	280±4	330±4
Probability	0.05	0.001
	------------ Relative (%) -------------	
High	0.53[b]	0.64[b]
Low	0.56[a]	0.67[a]
Mean±SEM	0.55±0.08	0.65±0.07
Probability	0.05	0.05

High or Low G eggs (Table 10), but plasma LDH was elevated at d 27 in High G embryos compared to Low, but not at hatching. Neither plasma glucose nor lactate concentrations differed between the two G treatments (Data not shown).

Embryos in eggs with High G hatched 6 hours earlier than did those in Low G eggs (Table 11). Low G embryos spent less time at internal pipping and more time at external pipping than did High. Thus, Low G hatched later than High G because of an extended time required to attain internal pipping and a longer time at external pipping.

Poults from each treatment were placed into brooders to determine the incidence of spontaneous cardiomyopthy (Paxton *et al.*, 2005). No differences were seen in the mortality rates in either females or males placed from the study.

Discussion

The current study shows clearly a direct relationship between G, k, embryo heart rates and survival. Highly significant positive correlations between G, k and heart rates were noted, and when the analysis included only

Table 7: Liver weight (g) of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	------------ Absolute (mg) ----------------	
High	1,121[a]	1,481
Low	1,023[b]	1,425
Mean±SEM	1,073±13	1,450±18
Probability	0.0007	NS
	--------------- Relative (%) -----------------	
High	2.21[a]	3.02[a]
Low	1.98[b]	2.76[b]
Mean ± SEM	2.09±0.02	2.89±0.03
Probability	0.0001	0.001

Table 8: Cardiac glycogen and lactate concentration (mg/g of wet tissue) of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	------------------ Glycogen ------------------	
High	1.71[b]	0.77
Low	2.15[a]	0.74
Mean±SEM	1.93±0.06	0.75±0.05
Probability	0.001	NS
	-------------------- Lactate -------------------	
High	1.62	1.67
Low	1.56	1.68
Mean±SEM	1.59±0.03	1.67±0.03
Probability	NS	NS
	--------------------- Ratio ---------------------	
High	1.08[b]	0.46
Low	1.42[a]	0.45
Mean±SEM	1.25±0.05	0.45±0.04
Probability	0.005	NS

Table 9: Hepatic glycogen and lactate concentration (mg/g of wet tissue) of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	----------------- Glycogen -------------------	
High	1.42	1.43
Low	1.83	1.22
Mean±SEM	1.63±0.20	1.32±0.10
Probability	NS	NS
	------------------- Lactate ------------------	
High	0.19[b]	0.22
Low	0.24[a]	0.23
Mean±SEM	0.22±0.07	0.22±0.01
Probability	0.001	NS
	--------------------- Ratio --------------------	
High	7.27[b]	6.59
Low	7.64[a]	5.55
Mean±SEM	7.45±0.80	6.08±0.45
Probability	0.005	NS

embryos that died, coefficients increased more than two-fold. Thus, embryos that died may have been in eggs with increased G that had rapid heart rates. Because

Table 10: Plasma creatine kinase (CK) and lactate dehydrogenase activities (LDH) (U/L) of turkey embryos developing in eggs with High and Low conductance (G)

G	Day of development	
	27	28
	----------------------- CK -----------------------	
High	1,448	2,567
Low	1,577	2,780
Mean±SEM	1,512±60	2,673±117
Probability	NS	NS
	----------------------- LDH -------------------	
High	523[a]	784
Low	507[b]	774
Mean±SEM	515±17	779±21
Probability	0.001	NS

coefficients were small, it suggests that only a small percentage of the decrease in heart rates can be explained in terms of G or k. Subsequently, eggs were coated with paraffin to reduce G and showed depressed heart rates. Thus, data from both experiments confirm a direct positive relationship between G and heart rates. Thus, as the porosity of the shell increases so does the embryo heart rate. Increased porosity and increased heart rates seem contradictory because embryos in eggs with low G would have a greater need for increased tissue oxygenation and heart rates.

Cardiac physiology and embryo survival were observed in the final experiment to determine if increased G and heart rates may affect the myocardium of developing embryos and their subsequent survival at the plateau stage. Embryos in High G eggs with accelerated heart rates died at a greater rate than did those in Low G eggs with reduced heart rates. This observation indicates that embryos that develop in High G eggs have a greater probability of dying with a more rapid heart rate. Thus, at least some embryo deaths may be caused by cardiac failure.

Perturbations in cardiac glycogen and lactate may be symptoms of cardiomyopathy in poults (Czarnecki and Evanson, 1980; Czarnecki, 1991; Liao *et al.*, 1996). Earlier studies of cardiomyopathic poults (Czarnecki *et al.*, 1975; Staley *et al.*, 1978; Czarnecki and Evanson, 1980; Mirsalimi *et al.*, 1990) noted excessive glycogen in various tissues. Glycogen granules were observed in lysozomes of cardiomyopathic turkeys which were hypothesized to result from a block in the citric acid cycle preventing the complete breakdown of glycogen and resulting in altered metabolism by the liver, including decreased protein synthesis and increased metabolism of fat, possibly associated with liver damage (Staley *et al.*, 1978). The best explanation for the altered levels was a change in degradation of glycogen (Czarnecki *et al.*, 1978). Conflicting reports exist, however, for cardiac glycogen in cardiomyopathic turkeys. One study reported

Table 11: Time to attain a stage of development and time at a stage (h) of turkey embryos developing in eggs with High and Low conductance (G)

G	IP Attain	IP At	EP Attain	EP At	Hatching
High	606[b]	18.2[a]	625	15.0[b]	640[b]
Low	611[a]	13.5[b]	624	22.1[a]	646[a]
Mean±SEM	609±1	15.9±0.9	624±1	18.6±0.8	643±1
Probability	0.05	0.03	NS	0.0004	0.0001

no change (Staley *et al.*, 1978), while another reported decreased levels (Mirsalimi *et al.*, 1990), but they did not account for the changes noted in heart size. Embryos in eggs with Low G in the current study had larger hearts, slower heart rates, and more glycogen than lactate in myocardium compared to High G. Thus, a better energy balance was present in Low G embryos than High, and those embryos survived the plateau stage better. Hepatic tissue also showed more glycogen than lactate in Low G embryos at pipping than did High G embryos. Our data agree with those or (Mirsalimi *et al.*, 1990) that imply that the glycogen to lactate ratios in heart were decreased but the ratios increased in the liver when myocardial health became limiting for embryos in High G eggs. The data contradict those of (Czarnecki *et al.*, 1978) and (Staley *et al.*, 1978).

A related line of research to examine cardiac health in poults involves studying CK, LDH and other enzymes involved in providing energy to the heart via the pathways of glycolysis and oxidative phosphorylation. Creatine phosphate carries a high-energy phosphate, which can be transferred to and from ATP by CK in a reversible reaction. It is a source of energy when other means of supplying ATP to cardiac muscle are inadequate (Liao *et al.*, 1996). Simply stated, significant decreases in the speed at which the phosphorylation of ATP from phosphocreatine were found in myocardium from cardiomyopathic turkeys. The amounts of both phosphocreatine and ATP were decreased while free carnitine levels were normal. ATP synthesis via oxidative phosphorylation did not appear to be impaired when concentrations of major marker enzymes were measured. These observations were correlated with decreased contractility of isolated cardiac muscle fibers, indicating that there may be a strong association between decreased energy reserve and decreased contractility in the failing heart, although decreases in ATP may not occur until late in pathogenesis (Liao *et al.*, 1996). CK activity was decreased 40% in myocardium of cardiomyopathic turkeys, the most of any of the energy related enzymes, including those involved in glycolysis (30% depression), the Kreb's cycle (20% depression), and fatty acid oxidation (15% depression) (Mirsalimi *et al.*, 1990). The CK activity in the current study was not depressed by High G, but LDH was elevated indicating a greater rate of Cori cycle activity recycling lactate into glucose. Thus, increased LDH implies that High G may have affected embryo cardiac health by decreasing cardiac muscle fiber energy.

The heart in embryos developing in Low G eggs was heavier, and the myocardium showed a better energy balance with more glycogen than lactate for muscular activity than did embryos in High G eggs. Thus, a possible explanation for the better survival in embryos with slower heart rates may be the stroke volume required to maintain blood supplies for growth and development of turkey embryos at the plateau in oxygen consumption (Dietz *et al.*, 1998). One contraction of the larger heart in an embryo with Low G may provide more oxygenated blood to growing tissues than does the more frequent beating of the smaller embryo heart in the High G egg with decreased glycogen to lactate ratios in the myocardium.

Prior data have indicated that shell thickness or G affected cardiac health (Christensen *et al.*, 2003). The shell provides the major resistance to vital gas diffusion to and from the embryo (Rahn, 1981) and the shell membranes may play a role early in development (Tullett and Board, 1976). Thus, thinner shells may facilitate increased G and vital gas exchange, but under the conditions of the current experiments, greater G reduced embryo survival.

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