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Effect of Incubator Temperature and Oxygen Concentration at the Plateau Stage in Oxygen Consumption on Turkey Embryo Muscle Growth and Development[1]

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Abstract: It was hypothesized that incubator temperature and oxygen concentration affect embryo muscle development. Turkey eggs were incubated until the 24th day of development. At the beginning of the 24th day, the eggs containing viable embryos were randomly divided into 4 groups immediately prior to the plateau stage in oxygen consumption. Four experimental cabinets accommodating approximately 100 eggs were used for the actual hatching process. Each cabinet operated at predetermined temperatures (TEM) and oxygen concentrations (O_2) in a 2 TEM (36° and 39°C) x 2 O_2 (17 and 23%) factorial arrangement. At 27 and 28 days of development, immediately following the plateau stage, 10 embryos or poults were sampled from each of the 4 cabinets. Blood was obtained following decapitation. From each carcass the pipping (*musculus complexus*), breast (*pectoralis thoracicus*) and thigh muscles (*gastrocnemus*) were collected. Muscles were placed into an appropriate volume of 7% perchloric acid preparatory to assaying for glycogen and lactate. Five birds were sampled for histological analyses of muscle fibers. Plasma Creatine Kinase (CK) and lactate dehydrogenase (LDH) activities were measured. TEM and O_2 affected muscle growth differently. High TEM and O_2 affected pipping and thigh muscle weights but not that of the breast muscle. Only TEM affected breast muscle weights. Muscle function was affected differently when embryos were exposed to TEM and O_2. The CK and LDH activities were also affected at 27 days 39°C causing elevated CK and LDH activities compared to 36°C. At 28 days, only CK was affected as 39°C elevated CK activity in the 23% oxygen environment but not in the 17% environment. Thus, incubator conditions may affect muscle development and function in poult embryos

Key words: incubator temperature, oxygen concentration, embryo muscle development

Introduction

Incubator temperature (TEM), oxygen (O_2) concentration (Metcalfe *et al*., 1981; Maltby and Strickland, 2004) and sex (Velleman and Nestor, 2004) may affect poult embryo muscle development. Development of the embryo muscle begins with the fusion of mononucleated myoblasts to form myotubes, which mature into myofibers (Shultz and McCormick, 1994). Feldman and Stockman (1992) showed that during avian development a distinct population of satellite cells is present at the midfetal stages of development and by late embryogenesis myoblasts found in the chicken embryo are predominantly adult myoblasts (Hartley *et al*., 1992). Maltby *et al.* (2004) showed the number of fibers in a turkey embryo may be increased by using increasing incubator temperature at mid embryonic stages. Once the bird is hatched and myofibers are formed, normal skeletal muscle growth occurs through an increase in myofiber size, rather than an increase in myofiber number (Remignon *et al*., 1995; Moore *et al*., 2005). Recently Velleman *et al.* (2002; 2003) reported that the inheritance of breast muscle is sex-linked and the dam is more responsible at specific ages than is the sire. Thus, sex may be an additional factor in muscle development of embryos. Moore *et al.* (2005) concluded that overall, early postnatal muscle development is the most critical time period for satellite cell mitotic activity. Little is known of the physiology and development of muscles in embryos when incubator conditions are not ideal. We proposed the hypothesis that incubator temperature, oxygen concentrations and embryo sex at the plateau stage in oxygen consumption may interact to affect muscle physiology and growth of thigh, pipping and breast muscles of developing turkey embryos.

Materials and Methods

Approximately 500 fertilized turkey eggs (Nicholas) were obtained from a commercial flock (Prestage Farms, Clinton, NC) for each of three experiments. The eggs were incubated (Natureform I14, Jacksonville, FL) for 24 days using standard industry procedures. The cabinet operated at a set point of 37.5°C dry bulb temperature and a RH of 53%. Eggs containing viable embryos on the 24th day of development were randomly divided into four groups immediately prior to the plateau stage in oxygen consumption. Four experimental cabinets accommodating approximately 100 eggs were used for the hatching process. Each cabinet contained one incubator tray and was regulated by thermistors connected to microprocessors with temperature

sensitivity of±0.05°C. Humidity was controlled by a similar system using relative humidity sensors. Digital thermometers (Cox, Lexington, NC) were used with each cabinet to verify set point temperatures. Atmospheric gases were measured by using an infrared detector for carbon dioxide (Engelhard Ventostat 2001V, Iselin, NJ 08830) and electrochemical cells for oxygen (Teledyne, Los Angeles CA 90064), each with a sensitivity of±0.1%. Manually operated valves infused gases into each of the cabinets and the flow rate was adjusted based on the sensors to create the desired gas concentrations. Three experiments were conducted using the experimental cabinets. Experiment 1 and 2 were preliminary experiments to determine the effects of temperature or oxygen on muscle development. Experiment 3 examined the interactive effects on muscle of the most effective and least effective for affecting muscle weights due to temperature and oxygen treatments determined in Experiments 1 and 2.

Temperature: The eggs were moved to the treatment cabinet at the beginning of the 24th day of development. The 24th day for poult embryos is the beginning of the plateau stage in oxygen consumption (Rahn, 1981). All infertile eggs and nonviable embryos were removed prior to transfer to one of four experimental cabinets. At the beginning of the 24th day the eggs were incubated at one of the treatment TEM (36, 37, 38 or 39°C) to expose the embryos to different temperatures at the plateau stage.

Tissue sampling: Ten embryos or poults were selected randomly from each incubator at the end of the treatment period as embryos were externally pipping during the 27^{th} day or hatching on 28 days of development. The poults were decapitated and trunk blood was collected into a tube containing 10 mg EDTA. Poult body weights were recorded (nearest 0.1g) with and without yolk and muscles were dissected and weighed (nearest 0.01 mg) as quickly as possible. The blood was centrifuged (700 x g) for 15 min under refrigeration (4°C). The plasma was decanted and frozen (-22°C) for analysis of Creatine Kinase (CK) (Oliver, 1955) and lactate dehydrogenase (LDH) (Wacker *et al.*, 1956) activities that may be indicative of muscle oxygen debt (Wilson *et al.*, 1990; Velleman and Nestor, 2004). Each sample was sexed by visual inspection following dissection. From each sample the pipping (*musculus complexus*), breast (*pectoralis thoracicus*) and thigh muscles (*gastrocnemus*) were collected. The three muscles were weighed (nearest 0.01g) and placed into an appropriate volume (5 mL/g of muscle) of cold (4°C) 7% perchloric acid. The plasma samples were capped in storage vials and kept at 4°C until assayed for glycogen (Dreiling *et al.*, 1987) and lactate (Henry, 1968; Trinder, 1969). Ratios of glycogen to lactate in each muscle were calculated by dividing the total glycogen measured in a muscle by the total lactate measured in the same muscle.

In each of the treatments described above, the *pectoralis thoracicus* was harvested from an additional 5 poults per treatment and fixed overnight with buffered neutral formaldehyde. The following morning the tissues were dehydrated, washed and embedded in paraffin. Eight micron thick cross sections were cut on a microtome and adhered to glass slides. Tissues were dewaxed and stained using standard hematoxylin and eosin procedures. A Leica DMR (Leica Microsystems, Bannockburn, IL) microscope was used to observe the tissue sections. A Spot-RT CCD (Diagnostic Instruments Inc., Sterling Heights, MI) camera was used to capture images of each of the cross sections. A randomly selected area on each slide was selected to measure the diameter of 100 fibers/section.

Oxygen: Four O_2 concentrations were the treatments in the second preliminary trial. All procedures to the 24^{th} day of development were the same as in Experiment 1. The O_2 concentrations within the cabinets were 17, 19, 21 or 23% of the atmosphere. The fractional concentration at sea level (Raleigh, NC) corresponded to oxygen partial pressures of 129, 144, 160 and 175 mm Hg, respectively. Concentrations lower than ambient oxygen concentrations (20.9%) were maintained by infusing nitrogen gas into the cabinet at a rate that resulted in the desired concentration of 17 or 19% O_2. Concentrations were measured with an oxygen meter and flow rates from oxygen or nitrogen storage tanks were adjusted to maintain the desired oxygen level. Embryo samples were collected and analyzed as in Experiment 1.

Temperature and oxygen: The most and least effective TEM (36° and 39°C) and O_2 treatments (17 and 23%) for altered physiology and the reduction of muscle weight in the preliminary experiments were combined in a factorial arrangement for the third experiment. All treatments were as described previously. Fertilized eggs were again incubated 24 days when viable embryos were assigned randomly to one of the four cabinets. The conditions were TEM of 36°C with 17 or 23% O_2 and 39°C with 17 or 23% O_2 in a factorial arrangement. Birds were sampled identically to the previous experiments.

Statistical analysis: Data for all three experiments were analyzed using the general linear models procedure (SAS Inc., 1998). Experiments 1 and 2 were arranged as a four levels of TEM or O_2 treatments by two sexes (hens and toms) by two days of development (27 and 28 d) factorial. In Experiment 3, the data were arranged as two TEM by two O_2 concentrations by two sexes factorial. Means differing significantly were separated by the least

Table 1: Body and yolk weights (g) of turkey embryos exposed to different oxygen concentrations and temperatures during the plateau stage of development

Oxygen (%)	Temperature (°C)	27 day embryo	28 day poult
BW without yolk			
17	36	51.8	59.0
	39	54.5	49.9
23	36	53.9	59.0
	39	53.9	52.3
	Mean±SEM	53.5±0.6	55.1±0.4
Probabilities		NS	TEM = 0.0001
Temperature means	36		59.0[a]
	39		51.1[b]
Yolk			
17	36	15.4	9.1
	39	16.5	12.8
	Mean	15.9[a]	11.0[a]
23	36	13.7	7.2
	39	12.7	10.3
	Mean	13.2[b]	8.7[b]
	Mean±SEM	14.6±0.2	9.8±0.3
Probabilities		O_2 = 0.0002	O_2 = 0.0017
			TEM = 0.0001
Temperature means	36		8.1[b]
	39		11.5[a]

[a–b]Means in a column with different superscripts are significantly different ($p<0.05$)

square means procedure. Means in tables are least square means. All possible main and interaction effects were tested. All probabilities were based on $p<0.05$ unless otherwise noted.

Results

Temperature and oxygen trials: The 39°C TEM and 17% O_2 reduced pipping and thigh muscle weights compared to 36°C and 23% O_2, respectively. However, weights of breast muscles were not affected by O_2 as only TEM affected only breast muscle weights. To prevent redundancy in the paper, no data will be reported from the initial two trials because their objective was to provide effective TEM and O_2 values for affecting muscle growth and physiology. Data from the initial trial indicated that poults muscle growth and physiology responded maximally to 39 and 36°C. Thus, these two TEM were selected for the third trial. Data from the second trial indicated that 17 and 23% O_2 were the oxygen treatments that elicited the maximal responses of muscle growth and physiology. Thus, the 17 and 23% O_2 treatments were used in the third trial.

Temperature with oxygen: There were no significant effects due to sex as a factor or interacting with other factors in the third trial so no sex effects will be reported. Only TEM affected body weights as 36°C increased weights compared to 39°C at 28 d of development (Table 1). Both O_2 and TEM affected residual yolk mass as 17% O_2 and 39°C increased residual yolk weight compared to 23% O_2 and 36°C, but the two factors did not interact ($p>0.05$).

Muscle weights at external pipping (27d) were decreased by 23% O_2 compared to 17% and TEM and O_2 interacted at hatching to affect the pipping muscle weight (Table 2). Using higher incubator TEM in combination with 17% O_2 increased pipping muscle weights in the hatched poult. Embryo breast muscles were heavier at 27 days in 39°C than in 36°C, but no differences were noted in poults due to either O_2 or TEM At hatch. The thigh muscle weight was affected only at hatching as 23% O_2 increased weights compared to 17% and 39°C depressed weights compared to 36°C, but the two factors did not interact. The ratio of pipping muscle glycogen to lactate displayed an O_2 by TEM interaction at 27 days as 39°C decreased the ratio in the 23% O_2 compared to 36°C but not in the 17% cabinets (Table 3). No effects were seen at day 28. The ratio of breast muscle glycogen to lactate was depressed by higher TEM at 27 days and by greater O_2 concentration at day 28. O_2 and TEM interacted to affect glycogen to lactate ratios in poult thigh muscles. In a 23% O_2 environment, 36°C elevated glycogen to lactate ratios compared to 39°C, but in a 17% O_2 environment, the reverse was noted.

At 27 days 39°C elevated CK and LDH activities compared to 36°C. At 28 days, only CK was affected as 39°C elevated CK activity in the 23% O_2 environment but not in the 17% environment (Table 4). TEM and O_2 interacted to affect breast muscle fiber diameter in poults hatching from the treatments. When exposed to a 17% O_2 concentration, 39°C suppressed muscle diameter compared to 36°C. When exposed to 23% O_2, no differences were noted between the TEM treatments (Table 5).

Table 2: Muscle weights (% of body mass) of turkey embryos exposed to different oxygen concentrations and temperatures during the plateau stage of development

Oxygen (%)	Temperature (°C)	27 day embryo	28 day poult
Pipping muscle			
17	36	1.61	1.10[b]
	39	1.57	1.43[a]
	1.59[a]		
23	36	1.36	1.17[b]
	39	1.43	1.07[b]
	1.40[b]		
	Mean±SEM	1.49±0.01	1.20±0.03
Probabilities	O_2 = 0.0366	O_2 x TEM = 0.0015	
Breast muscle			
17	36	5.28	6.96
	39	5.88	6.73
23	36	5.53	6.58
	39	6.10	6.53
	Mean±SEM	5.70±0.04	1.20±0.03
Probabilities		TEM = 0.0004	NS
Temperature means	36	5.40[b]	
	39	6.00[a]	
Thigh muscle			
17	36	4.76	4.76
	39	4.71	4.50
	Mean		4.63[b]
23	36	4.89	4.95
	39	4.76	4.75
	Mean		4.85[a]
	Mean±SEM	4.78±0.05	4.73±0.06
Probabilities		NS	O_2 = 0.0584
			TEM = 0.0434
Temperature means	36		4.86[b]
	39		4.62[a]

[a–b]Means in a column with different superscripts are significantly different ($p<0.05$)

Table 3: Ratio of muscle glycogen (mg) to lactate (mg) of turkey embryos exposed to different oxygen concentrations and temperatures during the plateau stage of development

Oxygen (%)	Temperature (°C)	27 day embryo	28 day poult
Pipping muscle			
17	36	4.85[ab]	1.91
	39	5.14[a]	1.96
23	36	4.34[b]	2.06
	39	2.91[c]	1.47
	Mean±SEM	4.35±0.22	1.84±0.14
Probabilities		O x T = 0.0439	NS
	Breast muscle		
17	36	2.84	1.45
	39	1.73	1.30
	1.38[a]		
23	36	2.66	1.20
	39	1.40	1.02
	1.11[b]		
	Mean±SEM	2.16±0.04	1.24±0.06
Probabilities		T = 0.0001	O = 0.0234
Temperature means	36	2.75[a]	
	39	1.56[b]	
Thigh muscle			
17	36	2.81	1.20[b]
	39	2.99	1.55[a]
23	36	2.34	1.77[a]
	39	2.70	0.78[c]
	Mean±SEM	2.71±0.14	1.32±0.02
Probabilities		NS	O_2 x TEM = 0.0001

[a–c]Means in a column with different superscripts are significantly different ($p<0.05$)

Table 4: Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activities of turkey embryos exposed to different oxygen concentrations and temperatures during the plateau stage of development

Oxygen (%)	Temperature (°C)	27 day embryo	28 day poult
CK (U/L)			
17	36	1,256	2,662[ab]
	39	1,411	2,284[b]
23	36	1,155	2,529[ab]
	39	2,133	2,842[a]
	Mean±SEM	1,488±112	2,579±100
Probabilities		TEM = 0.0117	O_2 x TEM = 0.0289
Temperature means	36	1,206[b]	
	39	1,772[a]	
LDH (U/L)			
17	36	380	712
	39	421	658
23	36	368	685
	39	492	730
	Mean±SEM	415±13	696±25
Probabilities		TEM = 0.0018	NS
Temperature means	36	373[b]	
	39	456[a]	

[a–b]Means in a column with different superscripts are significantly different ($p<0.05$)

Table 5: Breast muscle fiber diameters of turkey embryos exposed to different oxygen concentrations and temperatures during the plateau stage of development

Oxygen	Temperature	Breast diameter (µm)
17	36	3.06[a]
	39	2.54[c]
23	36	2.72[b]
	39	2.73[b]
Mean±SEM	2.77±0.14	
Probabilities	TEM	0.0019
	O_2	0.7135
	TEM x O_2	0.0153

[a–c]Means in a column with different superscripts are significantly different ($p<0.05$)

Discussion

In avian embryos development of muscle fibers begins with the fusion of mononucleated myoblasts to form myotubes, which mature into myofibers (Shultz and McCormick, 1994). A distinct population of satellite cells is present at the midembryonic stages of development and by late embryogenesis myoblasts found in the chicken embryo are primarily adult myoblasts (Hartley *et al.*, 1992). Once the bird is hatched and myofibers are formed, normal skeletal muscle growth occurs through an increase in myofiber size, rather than an increase in myofiber number (Remignon *et al.*, 1995). Myofiber number does not increase during normal postnatal growth because myonuclei are post mitotic and cannot synthesize DNA (Stockdale and Holtzer, 1961). Maltby and Strickland (2004) suggested that incubator temperature at critical times of turkey embryo development may affect the number of muscle fibers. Thus, the number and size of fibers that a poult has when it hatches may be important in the overall muscle development of the bird at market age (Moore *et al.*, 2005). Inheritance of breast muscle is sex-linked in turkeys so the bird sex may be a determinant of muscle maturation at hatching as well (Velleman *et al.*, 2002, 2003). The hypothesis tested in the current research was that incubation temperature and oxygen concentrations and embryo sex at the plateau in oxygen consumption affect the formation of muscles and their physiology. The hypothesis was tested specifically at the plateau stage in oxygen consumption, a time when muscle tissue may lack sufficient nutrients (Dietz *et al.*, 1998; Christensen *et al.*, 1999).

In this study, incubator temperature and cabinet oxygen concentrations effects on the development of each of the three muscles were followed. The factors were primarily independent, so interactions of O_2 and TEM did not alter the growth and physiology of breast muscle. Each muscle reacted differently to the TEM and O_2 treatments. Temperature is the major regulatory factor in egg incubation and embryo development because of its effect on general growth and the coincidental impact on tissue formation (Romanoff and Faber, 1933). Temperature manipulation at various times throughout incubation (38.5°C during days 5-8 and days 9-12 of development) can alter turkey embryo muscle development (Maltby *et al.*, 2004). Maltby *et al.* (2004) suggested that the nuclei and muscle fiber numbers in the posthatch turkey could be increased by temperature and Maltby and Strickland (2004) reported that temperature manipulations affected the posthatch phenotype through the alteration of muscle establishment in the embryo, but the pathways seemed obscure. A lower temperature increased gene expression of muscle at days 16 and 17 of development; however, if temperatures were higher (38.5°C) the expression was delayed until following day 21. In the current study we saw no increase in fiber numbers, but 36°C compared to 39°C did increase breast muscle fiber diameter when the eggs were incubated in a hypoxic environment.

Little is known of the effect of oxygen on muscle cell expression, but it has been suggested that oxygen may be the primary determinant of body and organ growth in the embryo (Metcalfe *et al.*, 1981) and hypoxia can alter the carbohydrate metabolism of growth-selected turkeys differently than that of controls (Christensen *et al.*, 1999). The impact of temperature and oxygen on embryo development is highly important because of the proliferating pool of muscle cells available in late incubation. In the current study TEM higher than 38°C and O_2 concentrations below 21% at the plateau affected muscle growth and physiology. Further study will be required to determine the long term effects of TEM and O_2 on muscle growth and physiology.

Because of the decreased fractional percentage of air cell oxygen during the final stage of embryonic development (Rahn, 1981), the embryo relies primarily on anaerobic metabolism for energy. Blood glucose increases steadily during this stage and tissue glycogen stores decrease (Freeman, 1965). The glucose from maternal investment in eggs is minimal by this time (Hazelwood and Lorenz, 1959). Because the egg contains so little glucose, glycogen is synthesized and stored in muscles earlier in incubation as a source of glucose in preparation for the hypoxia of hatching (Freeman, 1965). Gluconeogenic processes provide the necessary carbohydrate for glycogen storage. The liver and kidney are essential to this process because they convert lactate into glucose-6-phosphate when blood glucose levels decline (Watford *et al.*, 1981). Heart and skeletal muscle in avian species lack the ability to recycle lactate (Watford *et al.*, 1981). Therefore, the liver and kidney are supply organs in this sense and both heart and skeletal muscles are demand organs. Anything that interferes with overall energy metabolism may affect skeletal muscle growth and physiology as well.

Previously it was shown that incubator temperature and oxygen at the plateau stage affect the cardiovascular and digestive systems (Christensen *et al.*, 2004ab). Our data may be the first to show effects on embryo muscle development and physiology at the plateau stage in oxygen consumption. We suggest that each muscle has a unique mechanism to respond to hypoxia or hyperoxia to control growth and physiology. An example of uniqueness can be seen by comparing the results of the pipping and breast muscles. Pipping muscle responded to both increased TEM and decreased O_2 by increasing its size and increasing its ratio of glycogen to lactate. Conversely, the breast muscle weight decreased and the ratio of glycogen to lactate declined. Several possibilities exist to explain these observations. One possibility may be the muscle types. One is primarily a fast-twitch whereas the other is a slow-twitch muscle and each muscle type may have a different oxygen requirement. A second explanation may be the activity of each muscle is very different at the various embryonic stages of development. The pipping muscle may be contracting whereas the breast muscle can be less active at a given stage of development. It is noteworthy that all muscles continued to grow through the plateau stage despite physiological perturbations.

Gluconeogenesis requires recycling of lactate via the Cori cycle or catabolism of existing tissues or additional catabolism of available nutrients in residual yolk (Donaldson and Christensen, 1991) and occurs differently in turkeys selected for increased growth (Christensen *et al.*, 1999). If greater maturation could be attained prior to hatching, the poult in modern-type turkeys may be better able to grow muscle and have full muscle function even in an oxygen deficient environment (Dietz *et al.*, 1998). Eggs incubated at 36°C with greater than 21% O_2 had more glycogen than lactate in their muscles indicating a positive energy balance in those groups. The presence of greater amounts of glycogen may indicate metabolic adjustments to environmental conditions that could lead to muscle damage.

Elevated CK and LDH activities are characteristic of anoxia and hypercapnia in animals followed by a concomitant increase in gluconeogenesis (Oliver, 1955; Henry, 1968). 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 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 muscles from cardiomyopathy turkeys. LDH catalyzes the reversible reaction between pyruvate and lactate that is essential in gluconeogenesis. Elevated CK and LDH have been suggested to be correlates of skeletal muscle damage (Wilson *et al.*, 1990; Velleman and Nestor, 2004). The 39°C TEM elevated both CK and LDH at pipping but CK was affected only by TEM at hatching. Both of these observations indicate that oxygen debt and rapid lactate recycling were occurring due to elevated TEM. It is also noteworthy that increased CK activity due to elevated TEM occurred at hatching only in the presence of 23% O_2.

In conclusion, we present the first evidence known to the authors showing the critical importance temperature and oxygen at the plateau stage of turkey embryo development for muscle development. Incubator temperatures and oxygen concentrations both affected growth and function of muscles. It is recommended for optimal muscle growth and function, turkey eggs should be incubated at less than 37°C and in greater than 21% oxygen at the plateau stage in oxygen consumption.

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[1]The mention of trade names in this publication does not imply endorsement of the products mentioned nor criticism of similar products not mentioned.