Eggshell temperature manipulations during incubation and in ovo injection of thyroxine are associated with a decreased incidence of cold-induced ascites in broiler chickens

O. Afsarian,[*,1] M. H. Shahir,[*] A. Lourens,[†] A. Akhlaghi,[‡] H. Lotfolahian,[§] A. Hoseini,[§] and N. Mousavi[#]

[*]*Department of Animal Science, University of Zanjan, 45195–313 Zanjan, Iran;* [†]*Wageningen University and Research Center, Livestock Research, PO Box 338, 6700 AH, Wageningen, the Netherlands;* [‡]*Department of Animal Science, Shiraz University, 71441-65186 Shiraz, Iran;* [§]*Animal Science Research Institute, 31466-18361 Karaj, Iran; and* [#]*Department of Animal Science, Islamic Azad University, Varamin- Pishva Branch, Varamin, Tehran, Iran*

ABSTRACT A hypothesis was tested that eggshell temperature manipulations during incubation and in ovo injection of thyroxine (T_4) would help their progeny chicks to better survive the ascites-inducing condition during the growing period. In experiment 1, a total of 4,800 hatching eggs was randomly arranged in a 2 × 4 factorial design (8 replicates of 75 eggs per treatment), in which the eggs were incubated at a constant eggshell temperature (EST) of 37.8°C throughout the incubation period (CON) or were exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation (EST manipulations; EST_M), and 4 treatment groups of 3 control groups (no injection; INJ_N, needle pricked; INJ_P, and sterilized distilled water injection; INJ_W) and one T_4 treatment group (injected with sterilized distilled water containing 65 ng of T_4; INJ_{T4}). In experiment 2, 240 one-day-old male broiler chicks from 2 temperature conditions and injection (INJ_N and INJ_{T4}) treatment groups were reared for 42 d in a completely randomized design with a 2 × 2 factorial arrangement. To induce ascites, all chicks were exposed to a 15°C room temperature from 14 d onwards. Results from experiment 1 showed that second-grade chicks and yolk sac weight were decreased, and body weight at hatch was increased in the EST_M and INJ_{T4} groups. Also, final body weight was increased in the EST_M group. Ascites mortality rate was decreased in the EST_M and INJ_{T4} groups. In the EST_M and INJ_{T4} groups, the red blood cell (RBC) and the packed cell volume (PCV) count were decreased. In conclusion, the results showed that the EST_M and INJ_{T4} treatments during incubation were associated with improved chick quality, productive performance of broilers, and a decreased incidence of cold-induced ascites in broiler chickens.

Key words: ascites, chick quality, cold stress, performance, thyroid

2017 Poultry Science 0:1–9
http://dx.doi.org/10.3382/ps/pex302

INTRODUCTION

The ascites syndrome, also known as pulmonary arterial hypertension, is a metabolic disorder in broilers that is initiated by the high oxygen requirement of rapidly growing tissues in meat-type chickens (Julian, 1993; Currie, 1999). Although methods including genetic selection (Pavlidis et al., 2007), feed restriction (Shlosberg et al., 1991), feeding prebiotics (Solis de los Santos et al., 2005), clenbuterol (Ocampo et al., 1998), coenzyme Q_{10} (Geng et al., 2004) and potassium bicarbonate in drinking water (Shlosberg et al., 1998), or maternal hyperthyroidism (Akhlaghi et al., 2012) might be ameliorative, alternative approaches to minimize the outbreak of this syndrome would be greatly beneficial.

Because the length of the broiler production cycle decreased about 60% within 40 yr, the incubation period has become a larger part of the total life span of a broiler chicken (Baghbanzadeh and Decuypere, 2008; Molenaar et al., 2011). Therefore, optimizing development and maturation during incubation is most important and will in turn optimize the development during the rearing period (Hulet et al., 2007). Literature has indicated that a high eggshell temperature (**EST**) (≥38.9°C) during the second half of incubation reduced chick quality and increased the percentage of second-grade chicks (Leksrisompong et al., 2007; Molenaar et al., 2011). However, a few studies have addressed the effects of reduced EST on post- hatch performance. Afsarian et al. (2016) periodically exposed hatching eggs for one h to an ambient temperature of 15°C, resulting in periodically lower EST of 25.5, 27.0, 32.5, and

Received April 10, 2017.
Accepted September 15, 2017.
[1]Corresponding author: omid.afsarian@znu.ac.ir

36.0°C, respectively, at d 11, 13, 15, and 17 of incubation for a maximum period of one hour. Periodically cooling of hatching eggs below 37.8° EST during the plateau phase improved chick quality and development and reduced post-hatch ascites mortality in chicks exposed to low environmental temperatures.

There are some ambiguities in literature concerning thyroid hormones' role in the incidence of ascites syndrome. Several researchers have reported that the use of thyroid hormones can cause ascites syndrome in broiler chickens (Buys et al., 1998), which has been attributed to the increased basal metabolic rate and oxygen consumption of tissues (Bobek et al., 1977). On the other hand, chickens with ascites syndrome are not capable of maintaining sufficient levels of thyroid hormones, and these birds show considerably lower concentrations (Luger et al., 2001). This study postulated that broiler chickens exhibit ascites syndrome due to hypothyroidism. Also, Akhlaghi et al. (2012) reported that triiodothyronine ($\mathbf{T_3}$) and thyroxine ($\mathbf{T_4}$) concentrations decreased in cold-exposed ascetic birds. These researchers showed that the addition of T_4 in drinking water of the breeder hens prevent reduction of T_4 levels in the cold-exposed chickens. Accordingly, ascites mortality rate has been reported to be associated with hyperthyroidism in broiler chickens in cold conditions (Luger et al., 2002). However, the development of embryos is closely associated with metabolic status. In other words, although the shortage of oxygen supply in ascetic birds seems to be related to cardiovascular complications, the role of thyroid hormones in the development of embryonic organs, especially the lungs, should not be ruled out.

As far as we know, there are no reports on in ovo injection of T_4 during incubation. Also, the association between in ovo injection of T_4 and the plausible decreasing effect on ascites incidence in broiler chickens has not been reported previously. Moreover, there is an unclear relationship between EST manipulation during incubation and in ovo injection of T_4 on the thermal resistance and the incidence of ascites in broiler chickens grown in a cold ambient condition. Therefore, the aim of the present study was to evaluate the effects of EST alteration and in ovo injection of T_4 on the incidence of ascites syndrome caused by post-hatch low temperature exposure in broiler chickens.

MATERIALS AND METHODS

Experimental Design

Two experiments were performed hypothesizing that low EST manipulation during incubation and in ovo injection of T_4 would help their progeny chicks to better survive ascites-inducing conditions during the growing period. The first experiment ended at hatching when embryonic mortality, chick quality, and hatchability were determined. In the second experiment, the chicks were reared until slaughtering at 42 d of age when body weight (**BW**), feed intake (**FI**), feed conversion ratio (**FCR**), and mortality were determined, as well as ascites mortality, immune cell count, and thyroid hormones status. This experiment was conducted under a protocol approved by the Animal Care and Welfare Committee of our institute.

Incubation (Experiment 1)

A total of 6,000 hatching eggs of approximately similar weights (65 ± 6.5 g) was collected from a 36-week-old Arian breeder flock (Babolkenar Arian Line Breeding Center, Babolkenar, Iran). The eggs were stored for 3 d under standard conditions (18°C and 65% relative humidity) and then incubated in a single incubator with a total setting capacity of 16,800 eggs (Petersime, Zulte, Belgium). The eggs were incubated at 37.8°C machine temperature and at a relative humidity of 55 to 60% during the first 10 d of incubation. On d 11 of incubation, the eggs were candled as specified by Ernst et al. (2004), and infertile eggs, cracked eggs, or eggs containing dead embryos were removed and discarded. After candling, a total of 4,800 hatching eggs was randomly allotted to 8 treatment groups. Each group consisted of 8 replicates of 75 eggs (600 eggs/treatment group in total). This experiment was performed in a 2 × 4 factorial arrangement with incubation temperature group and injection process as factors. The eggs were further incubated at a constant EST of 37.8°C throughout the incubation (**CON**) or exposed to 15°C machine temperature for one h on d 11, 13, 15, 17 of incubation (eggshell temperature manipulations; $\mathbf{EST_M}$). Next, eggs in each temperature treatment were subjected to one of the following treatments: no injection ($\mathbf{INJ_N}$), needle- pricked ($\mathbf{INJ_P}$), sterilized distilled water injection (0.5 mL; $\mathbf{INJ_W}$), or T_4 injection (0.5 mL of sterilized distilled water containing 65 ng of T_4; $\mathbf{INJ_{T4}}$).

All injected solutions were prepared on the d of injection using distilled water. T_4 (Iran Hormone Drug Co., Tehran, Iran) was dissolved in the diluent to achieve the final concentration of 65 ng per injection. The diluent and the T_4 solution were then autoclaved at 121°C for 15 min and were subsequently allowed to cool down to 37.8°C before injection. The volume of injections was set at 0.5 mL for all treatment groups. The injection practice was done on d 18 of incubation by using of a syringe equipped with a 27-gauge needle. Injection depth was approximately 2.5 cm from the top of the blunt end of the egg. The detailed injection procedure was described previously by Zhai et al. (2011a;b) and Ebrahimi et al. (2012).

At hatching, a total of 16 chicks per treatment was randomly selected to determine chick weight and then killed by cervical dislocation to measure yolk free body weight ($\mathbf{BW_{YF}}$) and yolk sac weight (**YW**). After hatching, the chicks were classified as first or second grade, for which the chicks that were clean and without

lesions or deformities were classified as first grade and other chicks were classified as second grade (Molenaar et al., 2011). The rates of embryonic mortality, hatchability, and culled chicks were expressed as a percentage of fertile eggs (Molenaar et al., 2011). Unhatched eggs were opened to determine the fertility and stage of embryonic mortality at zero to 3 d, 4 to 14 d, 15 to 18 d, or 19 to 21 d, according to Hamburger and Hamilton (1951).

Grow-out (Experiment 2)

A total of 240 male chicks from incubation temperature (CON and EST_M) and injection process (INJ_N and INJ_{T4}) groups was selected for a 42-day grow-out period. Room temperature was set at 32°C on the first d of age and decreased to 23°C gradually by 3°C/wk until 21 d of age. Afterwards, ascites-inducing conditions were provided according to Akhlaghi et al. (2012). All groups were reared under 15°C until the end of the experiment (between 22 and 42 d). The chicks were exposed to 23 h light and one h darkness through the end of the study. The study was conducted in a 2 × 2 factorial arrangement with incubation temperature (CON and EST_M) and injection process (INJ_N and INJ_{T4}) groups as factors. Each replicate was reared on a floor pen (1.5 × 1.5 m) covered with wood shavings. The birds were randomly assigned to the replicates (4 replicates of 15 chicks per treatment) and were fed a conventional mash broiler starter diet (22% CP and ME 2,850 kcal/kg of diet) between d one to 13, grower diet (21.1% CP and ME 2,896 kcal/kg of diet) between d 14 to 28, and a finisher diet (19.1% CP and ME 2,958 kcal/kg of diet) between d 29 to 42. The diets met or exceeded the strain requirement as appropriate. Feed and water were provided ad libitum throughout the study.

The BW was monitored on a weekly basis. The FCR was determined on a pen basis using the weekly BW and feed consumption values. At 42 d of age, blood samples were taken from the brachial vein into EDTA-coated tubes. Approximately 1 mL of blood was taken for determination of red blood cells (**RBC**) and packed cell volume (**PCV**). RBC were counted in a hemocytometer chamber using Natt and Herrick's solution to obtain a 1:200 blood dilution (Maxwell et al., 1986). PCV percentage was determined by centrifugation of microhematocrit capillary tubes at 1,800 × *g* for 12 min at room temperature (Schalm et al., 1975). Also, 0.5 mL of blood was taken from each bird and used to prepare a blood smear slide. The slides were stained using a hematology staining kit (Polysciences Inc, Warrington, PA), air dried, and stored in a slide box. Heterophil/lymphocyte ratio was measured under 1,000 × magnifications using light microscopy.

Another blood portion was centrifuged (12 min at 1,800 × *g*), and the plasma was stored at − 20°C for further analysis (Tankson et al., 2002). Plasma T_3 and T_4 concentrations were analyzed using commercially available kits (Pars Azmon, Tehran, Iran) validated for blood plasma of chickens (Akhlaghi et al., 2012). Briefly, samples were diluted at a rate of 1 to 5 with the dilution buffer to validate for parallelism and recovery rate. The concentrations of T_3 and T_4 were calculated from a standard curve, ranging between 0.5 to 300.0 and 0.03 to 10.0 ng/mL for T_3 and T_4, respectively ($r > 0.99$). Using a calibrator solution (T_4 = 0.00 ng/mL), serial dilutions (at the ratios of 1:2, 1:4, 1:8, and 1:16) were made for 4 plasma samples with known T_4 concentrations to control for linearity. Each sample was evaluated in duplicate. The mean recovery rates were 90, 96, 103, and 109% for the dilution rates of 1:2, 1:4, 1:8, and 1:16, respectively. This procedure also was implemented for T_3 with recovery rates of 92, 95, 101, and 104% for the serially diluted samples. Mortality rate was recorded daily and necropsies performed daily for diagnosis of ascetic birds (Luger et al., 2002). The ascites incidence was presented as the cumulative ascites mortality throughout the production period, plus ascites observed at necropsy on d 42. At the end of rearing, 8 birds per treatment were killed to weigh the heart, liver, spleen, and bursa of Fabricius.

Statistical Analysis

The data were analyzed using SAS (SAS Institute, 2002). Analyses for percentage data, including hatchability, embryonic mortality, and chick quality, were conducted after square root of arc-sine transformation of data. Significant differences between treatment means were determined by the Duncan's multiple range tests. Data from the rearing period of experiment 2 were analyzed using the general linear model (**GLM**) procedure. Total mortality and mortality due to ascites were analysed with a logistic regression model by the GENMOD procedure of the same package. Differences among treatments were considered by the Duncan's multiple range tests. In all cases, a difference was considered significant at $P \leq 0.05$.

RESULTS

The EST was more influenced in young embryos than in older embryos. Therefore, reducing temperature at d 17 of incubation had only a small effect on the EST (see Afsarian et al., 2016). The hatchability of fertile eggs was not affected by experimental treatments; however, the percentage of first-grade chicks was increased by EST_M and INJ_{T4} treatments (Table 1). Embryonic mortality rate in the third wk of incubation was significantly increased by EST_M ($P = 0.01$; Table 1).

The BW and BW_{YF} at hatching were higher in the EST_M treatments than in the CON (Table 2). Also, YW in EST_M ($P = 0.03$) and INJ_{T4} ($P = 0.001$) was lower as compared to the control treatments (Table 2).

Final BW at the end of the rearing period in the EST_M group was 126 g higher than that recorded for

Table 1. Effects of eggshell temperature (EST) manipulations during incubation and in ovo injection of T_4 on hatchability, chick quality, and embryonic mortality.[1]

Item	Hatchability[2]	First-grade chicks[2]	Embryonic mortality (d)[2]			
			0 to -3	4 to -14	15 to -18	19 to -21
EST						
CON	79.51	78.42[b]	5.60	4.20	2.99[b]	7.79
EST_M	80.89	82.72[a]	5.14	3.95	4.51[a]	5.50
SEM	*1.45*	*0.27*	*0.48*	*0.46*	*0.42*	*1.02*
INJ						
INJ_N	84.69	79.03[b]	4.61	3.71	2.64	4.47
INJ_P	78.12	78.33[b]	6.00	5.12	3.75	7.00
INJ_w	79.25	76.88[b]	5.25	2.87	3.87	8.75
INJ_{T4}	78.75	88.04[a]	5.62	4.50	4.75	6.37
SEM	*2.04*	*2.06*	*0.67*	*0.65*	*0.59*	*1.44*
EST × INJ						
CON × INJ_N	84.05	76.07	4.66	3.89	1.22	6.42
CON × INJ_P	78.50	77.05	6.50	4.75	3.25	7.00
CON × INJ_w	79.75	75.44	5.25	3.75	3.50	8.25
CON × INJ_{T4}	76.25	85.11	6.00	4.25	4.00	9.50
EST_M × INJ_N	85.33	82.00	4.57	3.54	4.05	2.51
EST_M × INJ_P	77.75	79.60	5.50	5.50	4.24	7.00
EST_M × INJ_w	79.25	78.32	5.25	2.00	4.25	9.25
EST_M × INJ_{T4}	81.25	90.96	5.25	4.75	5.50	3.25
SEM	*2.67*	*2.91*	*0.95*	*0.93*	*0.83*	*2.03*
Source of variation						
EST	0.50	0.05	0.50	0.75	0.01	0.12
INJ	0.10	0.01	0.52	0.10	0.10	0.22
EST × INJ	0.76	0.07	0.94	0.53	0.61	0.26

[a,b]Within column for each effect, values with different superscripts differ significantly ($P \leq 0.05$).

[1]CON = eggs incubated at 37.8°C EST; EST_M = eggs exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation; INJ_N = no injection; INJ_P = needle pricked; INJ_W = sterilized distilled waterinjection; INJ_{T4} = T_4 injection.

[2]As a percentage of fertile eggs.

Table 2. Effects of eggshell temperature (EST) manipulations during incubation and in ovo injection of T_4 on chick quality indices.[1]

Item	BW (g)[2]	BW_{YF} (g)[2]	YW (%)[2]
EST			
CON	47.03[b]	38.59[b]	8.68[a]
EST_M	48.50[a]	40.66[a]	7.84[b]
SEM	*0.51*	*0.47*	*0.27*
INJ			
INJ_N	48.75	40.44	8.69[a]
INJ_P	47.75	39.50	8.80[a]
INJ_w	47.18	38.81	8.72[a]
INJ_{T4}	47.37	39.75	6.83[b]
SEM	*0.73*	*0.67*	*0.39*
EST × INJ			
CON × INJ_N	47.50	38.62	9.30
CON × INJ_P	47.12	38.25	9.55
CON × INJ_w	47.25	38.62	9.49
CON × INJ_{T4}	46.25	38.87	6.39
EST_M × INJ_N	50.00	42.25	8.10
EST_M × INJ_P	48.37	40.75	8.05
EST_M × INJ_w	47.12	39.00	7.60
EST_M × INJ_{T4}	48.50	40.62	7.27
SEM	*1.03*	*0.95*	
Source of variation			
EST	0.05	0.03	0.03
INJ	0.44	0.40	0.001
EST × INJ	0.57	0.38	0.10

[a,b]Within column for each effect, values with different superscripts differ significantly ($P \leq 0.05$).

[1]CON = egg incubated at 37.8°C EST; EST_M = egg exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation; INJ_N = no injection; INJ_P = needle pricked; INJ_W = sterilized distilled water injection; INJ_{T4} = T_4 injection.

[2]BW = Body weight; YW = relative weight of yolk sac; BW_{YF} = yolk free body weight.

the CON treatment group ($P = 0.01$; Table 3). The FCR was decreased in the EST_M ($P = 0.05$) and INJ_{T4} ($P = 0.02$) treatments as compared to the control treatments (Table 3). Ascites mortality rate in the EST_M ($P = 0.01$) and INJ_{T4} ($P = 0.05$) treatments groups were 6.67 and 8.12 percent, respectively, being as low as that found in the CON groups (Table 3).

Relative spleen weight was greater in the EST_M ($P = 0.03$) and INJ_{T4} ($P = 0.01$) than in the CON group treatments. Also, the number of lymphocytes in the EST_M group ($P = 0.01$) and the number of heterophils in INJ_{T4} ($P = 0.04$) were, respectively, increased and decreased, in comparison with the CON groups. Heterophil/lymphocyte ratio was lower in the EST_M ($P = 0.02$) and INJ_{T4} ($P = 0.03$) treatments (Table 4).

Heart weight, RBC, PCV, and the plasma levels of T3 and T4 were lower in the EST_M and INJ_{T4} treatments than in the CON groups ($P < 0.05$; Table 5).

DISCUSSION

The aim of the experiment was to determine the effects of eggshell temperature manipulation during incubation and in ovo injection of T_4 on hatchability and chick quality on hatching d, as well as broiler performance and ascites incidence in later life. The manipulation of EST and in ovo injection of T_4 exerts a positive effect on the development of organs and chick quality at the time of hatch. According to the

Table 3. Effects of eggshell temperature (EST) manipulations during incubation and in ovo injection of T_4 on growth performance and ascites mortality.[1]

Item	FI (g)[2]	BW (g)[2]	FCR[2]	Ascites mortality (%)	Overall mortality (%)
EST					
EST_M	3986	2,090[a]	1.91[b]	6.67[b]	13.33
CON	3933	1,964[b]	2.01[a]	14.63[a]	25.75
INJ					
INJ_{T4}	3903	2061	1.90[b]	8.12[b]	16.25
INJ_N	4017	1993	2.02[a]	13.17[a]	22.83
SEM	*47.32*	*29.02*	*0.031*	*1.68*	*4.41*
EST × INJ					
$EST_M \times INJ_{T4}$	3963	2104	1.89	5.00	10.00
$EST_M \times INJ_N$	4009	2077	1.93	8.33	16.67
CON × INJ_{T4}	3841	2018	1.91	11.25	22.50
$EST_M \times INJ_N$	4024	1910	2.10	18.00	29.00
SEM	*66.91*	*41.04*	*0.044*	*2.38*	*6.23*
Source of variation					
EST	0.44	0.01	0.05	0.01	0.07
INJ	0.11	0.12	0.02	0.05	0.31
EST × INJ	0.32	0.35	0.09	0.49	0.99

[a,b]Within column for each effect, values with different superscripts differ significantly ($P \leq 0.05$).

[1]CON = egg incubated at 37.8°C EST; EST_M = egg exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation; INJ_N = no injection; INJ_{T4} = T_4 injection.

[2]FI = feed intake; BW = body weight; FCR = feed conversion ratio.

Table 4. Effects of eggshell temperature (EST) manipulations during incubation and in ovo injection of T_4 on immune organ relative weight and immune cell count.[1]

Item	Spleen (%)	Bursa of Fabricius (%)	Leukocyte (× 10^3)	H (%)[2]	L (%)[2]	H/L[2]
EST						
EST_M	0.159[a]	0.166	28.11	26.50	70.62[a]	0.37[b]
CON	0.130[b]	0.151	28.31	27.62	68.25[b]	0.40[a]
INJ						
INJ_{T4}	0.161[a]	0.165	28.05	26.12[b]	69.37	0.38[b]
INJ_N	0.128[b]	0.153	28.37	28.00[a]	69.50	0.40[a]
SEM	*0.08*	*0.019*	*0.71*	*0.58*	*0.54*	*0.01*
EST × INJ						
$EST_M \times INJ_{T4}$	0.165	0.185	27.30	26.00	70.00	0.37
$EST_M \times INJ_N$	0.153	0.148	28.92	27.00	71.25	0.38
CON × INJ_{T4}	0.158	0.145	28.80	26.25	68.75	0.38
$EST_M \times INJ_N$	0.103	0.158	27.82	29.00	67.75	0.43
SEM	*0.012*	*0.026*	*1.00*	*0.83*	*0.77*	*0.011*
Source of variation						
EST	0.03	0.58	0.84	0.20	0.01	0.02
INJ	0.01	0.64	0.75	0.04	0.87	0.03
EST × INJ	0.09	0.36	0.22	0.31	0.17	0.11

[a,b]Within column for each effect, values with different superscripts differ significantly ($P \leq 0.05$).

[1]CON = egg incubated at 37.8°C EST; EST_M = egg exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation; INJ_N = no injection; INJ_{T4} = T_4 injection.

[2]H = Heterophil percentage; L = lymphocyte percentage; H/L = Heterophil/lymphocyte ratio.

literature, the absorption of yolk sac contents to the abdominal cavity of the fetus occurs immediately before hatching and is influenced by the level of T_4 hormone (Wilson and McNabb, 1997). Gradual increase in the secretion of this hormone from the thyroid gland of the fetus stimulates growth and development of the fetus and provides an optimum condition for subsequent life, such as absorption of the yolk sac (McNabb, 2006). Based on the results of the present study, the number of first-grade chicks was increased in INJ_{T4} group compared to the INJ_N. To the best of our knowledge, this is the first study investigating the effect of in ovo injection of T_4 on chick quality—respectively, 88.04 vs. 79.03% for chickens in the INJ_{T4} and INJ_N groups.

According to results of this study, EST_M during incubation increased percentage of first-grade chicks. This result is in line with the studies proving the effectiveness of EST on yolk sac absorption and fetal development (Lourens et al., 2005, 2007; Maatjens et al., 2014). The fetus is a poikilothermic organism and has limited ability to regulate its body temperature by means of increased or decreased heat production during incubation (Romjin and Lokhorst, 1955). Therefore, metabolic rate of the fetus is mainly influenced by temperature (Maatjens et al., 2014). It has been reported that the reduction in EST increases metabolic rate and heat production in the fetus (Afsarian et al., 2016). Therefore, it can be speculated that the EST_M can improve the absorption of the yolk sac at the time of hatch.

Table 5. Effects of eggshell temperature (EST) manipulations during incubation and in ovo injection of T_4 on visceral organs, RBC, PCV, and thyroid hormones status of broilers at 42 d of age.[1]

Item	Heart (%)	Liver (%)	RBC ($\times 10^{6/\mu}$L)	PCV (%)	T_3 (*ng*/mL)	T_4 (*ng*/mL)	T_3/T_4
EST							
EST_M	0.553[b]	2.47	2.36[b]	29.90[b]	2.64[b]	4.91[b]	0.55
CON	0.609[a]	2.50	2.52[a]	32.35[a]	3.60[a]	6.28[a]	0.59
INJ							
INJ_{T4}	0.555[b]	2.48	2.36[b]	30.25[b]	2.80[b]	4.89[b]	0.58
INJ_N	0.606[a]	2.48	2.52[a]	32.00[a]	3.41[a]	6.29[a]	0.55
SEM	*0.017*	*0.12*	*0.052*	*0.56*	*0.20*	*0.44*	*0.04*
EST × INJ							
$EST_M \times INJ_{T4}$	0.555	2.39	2.32	29.85	2.53	4.67	0.55
$EST_M \times INJ_N$	0.550	2.54	2.40	29.95	2.74	5.14	0.54
$CON \times INJ_{T4}$	0.555	2.57	2.41	30.65	3.06	5.12	0.62
$EST_M \times INJ_N$	0.663	2.42	2.63	34.05	4.07	7.43	0.56
SEM	*0.017*	*0.17*	*0.074*	*0.80*	*0.28*	*0.62*	*0.06*
Source of variation							
EST	0.04	0.88	0.05	0.01	0.01	0.05	0.46
INJ	0.05	0.98	0.05	0.05	0.05	0.04	0.56
EST × INJ	0.04	0.41	0.40	0.06	0.18	0.17	0.70

[a,b]Within column for each effect, values with different superscripts differ significantly ($P \leq 0.05$).

[1]CON = egg incubated at 37.8°C EST; EST_M = egg exposed to 15°C for one h on d 11, 13, 15, and 17 of incubation; INJ_N = no injection; INJ_{T4} = T_4 injection.

The incubation temperature affects organ development and chick quality at the time of hatch (Ipek et al., 2014). It has been proved that the quality of day-old chickens is a prerequisite for the start of a successful breeding period and later chick performance (Willemsen et al., 2008; Meijerhof, 2009). However, high BW_{YF} is one of the most important indices in assessing chick quality (Lourens et al., 2005; Joseph et al., 2006; Maatjens et al., 2014). In the present study, chickens in the EST_M group showed the highest BW and BW_{YF} and the lowest YW on the d of hatch. This is in agreement with the results of previous studies (Joseph et al., 2006; Yalçın et al., 2012; Afsarian et al., 2016), which concluded that the reduction in temperature during later stages of the incubation period decreases yolk sac weight and increases chick BW through positive effects on the fetus. It seems that decreased YW and increased BW_{YF} at the time of hatch are associated with the establishment of a balance in energy expenditure for survival or the development of muscles and organs.

YW was decreased in the INJ_{T4} group compared to the INJ_N group. Generally, yolk sac consumption reaches 1 gr/d during the last 2 d of incubation due to a peak in T_4 secretion (Noble, 1987). It seems that 21% decrease in yolk sac weight at the time of hatch after in ovo injection of T_4 is related to the increasing effect of this hormone on metabolism.

Changes in incubation temperature can influence the economic efficiency of the broiler breeding industry via affecting post-hatch performance (Lourens and van Middelkoop, 2000; Lourens et al., 2005). At the end of the breeding period, chickens in the EST_M group weighed more than the CON group. Previous research showed that higher BW of chickens at the time of hatch affects slaughter weight, although this relationship seems uncertain (Tona et al., 2003 and 2004). Anyway, Shahir et al. (2012) and Shinder et al. (2002, 2009) reported that metabolism, feed consumption, and BW can be influenced by thermal stimulation at the embryonic stage or the first wk of life. Thermal stimulation in the embryonic period can stimulate the hypothalamus-hypophysis-thyroid axis and increase fetal T_4 secretion (McNabb, 2006 and 2007), which in turn regulates metabolic rate and affects development of organs, such as the intestine (Akhlaghi et al., 2013). Some reports show that high levels of T_4 in the embryonic period improve the absorptive capacity of the intestine (Van der Geyten et al., 2002; Akhlaghi et al., 2013). In addition, Afsarian et al. (2016) reported that administration of periodical low EST during incubation prevents later increase in thyroid hormone levels in broiler chickens kept in cold conditions. Therefore, the lack of changes in metabolic rate of a bird exposed to cold weather improves its performance under cold stress. FCR was improved in the INJ_{T4} group compared to the INJ_N group, which may be associated with development of the intestine at the time of hatch (Wasan et al., 2005; Molenaar et al., 2011; Akhlaghi et al., 2013). Palo et al. (1995) reported that the development of the gastrointestinal tract has a major role in the growth of broilers during the post-hatch period. As a product of intestinal deiodination of T_4, T_3 plays an important role in differentiation of the intestine during the last wk of incubation (Van der Geyten et al., 2002). In ovo injection of T_4 can increase T_3 production and thus improve differentiation and development of the intestine. Therefore, the improved absorptive performance of the intestine and bird yield can be expected.

Ascites is the main cause of mortality in broiler breeding units throughout the world (Wideman and French, 2000; Akhlaghi et al., 2012). Low temperature and high growth rate during the breeding period are major underlying factors for the incidence of the ascites due to their increasing effect on metabolic rate and oxygen demand (Wideman, 2001; Baghbanzadeh and Decuypere, 2008). Therefore, we decreased the ambient

temperature in order to induce ascites in the present study. Ascites-induced mortalities were reduced in the EST_M group compared to the CON, which was consistent with the results of Shahir et al. (2012) and Shinder et al. (2011), who reported decreased ascites-induced losses in chicks exposed to cold conditions in the embryonic stage or early stages of the breeding period. Lower incidence of ascites in the EST_M group can be associated with metabolism of thyroid hormones in birds under cold stress (Afsarian et al., 2016). These researchers reported that a periodical low EST during incubation prevents elevation of thyroid hormone levels in broilers exposed to lower environmental temperatures. Therefore, the lack of an increase in thyroid hormone concentration can prevent ascites.

Also, according to the results of the present study, ascites-induced mortalities were decreased in the INJ_{T4} group compared to the INJ_N. This can be related to the effect of thyroid hormones on the development of the vascular system and increased efficiency of O_2 and CO_2 exchange. Wittmann et al. (1983) reported that the thyroid hormone is necessary for the maturity of the lungs in birds. In addition, Van der Geyten et al. (2002) reported that the production of T_3 through deiodination of T_4 in the lungs affects the cardiovascular system. Therefore, it appears that in ovo injection of T_4 leads to optimal maturation of the respiratory and cardiovascular systems in broiler chickens. However, further research is needed to confirm this hypothesis.

According to the results of this study, the relative weight of the spleen and the percentage of lymphocytes were higher in the EST_M group than the CON group. This finding contradicts that of Santin et al. (2003), who suggested that changing the incubation temperature did not affect immune responses. The difference found in these 2 experiments may be attributed to duration of exposure to cold, degree of cold stress, or the genetic background of the birds.

The increase in the relative weight of the spleen in birds of the INJ_{T4} group might be due to the reduction in stressful factors such as temperature change or stock density during the breeding period, since these factors increase secretion of corticosteroids from the adrenal glands. These compounds can decrease relative weight of the thymus and spleen, cell proliferation factor, and interleukin II (Siegel and Latimer, 1984). Therefore, it seems that the use of T_4 during the embryonic stage reduces stress and increases resistance of the birds to stress under cold conditions in later life. Also, the number of heterophils was decreased in the INJ_{T4} group compared to the INJ_N. Siegel and Latimer (1984) found that the increase in corticosteroid secretion from the adrenal gland increased the number of heterophils and lymphocytes. Therefore, the reduction in heterophils in the INJ_{T4} group can be associated with changes in secretion of these hormones.

The heterophile/lymphocyte ratio is an appropriate index of stress in broiler chickens (Puvadolpirod and Thaxton, 2000). Therefore, a lower heterophile/lymphocyte ratio in the EST_M and INJ_{T4} groups is indicative of resistance to reduction or changes in temperature among these birds and that these birds may not sense reduction in ambient temperature.

In the present study, relative weight of the heart, RBC count and PCV were lower in the EST_M group compared to the CON group. Yahav et al. (1997), Balog et al. (2003), Kamely et al. (2015) and Julian (1987) reported a direct relationship between reduction in temperature and increased relative weight of the heart and viscosity of blood. Generally, with the exposure of chickens to low temperatures, the demand for oxygen increases over 185% (Gleeson, 1986). Therefore, heart rate enhances in order to increase cardiac output and to compensate for the shortage of oxygen in tissues, leading to elevated heart muscle weight. On the other hand, erythropoiesis increases to control hypoxia and subsequently increases the number of RBC, resulting in lower oxygen delivery and increased blood viscosity (Rajani et al., 2011). Since birds did not sense the reduction in ambient temperature in the present study, we did not observe any increase in heart relative weigh, RBC, or PCV.

Relative weight of the heart, RBC, and PCV were decreased in INJ_{T4} group compared to the INJ_N, which was consistent with the result obtained by Luger et al. (2002), who reported decreased hematocrit and RBC in chickens receiving T_4 and kept in cold conditions. This may be related to the effect of T_4 on the development of the lungs in the embryonic stage.

The results of the present experiment showed that levels of T3 and T4 in chickens in the CON group were higher, compared to the EST_M group. This finding was consistent with the result obtained by Shahir et al. (2012), who reported that cold conditioning of broiler chicks decreased the circulatory T3 concentration.

In the present study, the level of thyroid hormones was lower in the INJ_{T4} group than the INJ_N. Generally, thyroid hormones influence heat-sensing neurons of the hypothalamus in the embryonic stage and alter the sensitivity of these neurons to temperature changes during the rearing period (McNabb, 2006, 2007). Therefore, it can be speculated that the use of an exogenous T_4 in the embryonic stage can influence this process, and environmental temperature reduction does not change the secretion of thyroid hormones in chickens of the INJ_{T4} group.

In conclusion, the results of the present study showed that the manipulation of EST on d 11, 13, 15, and 17 of incubation as well as in ovo injection of T_4 influence chick quality and performance during the breeding period.

REFERENCES

Afsarian, O., M. H. Shahir, A. Akhlaghi, H. Lotfolahian, A. Hoseini, and A. Lourens. 2016. Periodical low eggshell temperatures during incubation and post hatch dietary arginine supplementation: Effects on performance and cold tolerance acquisition in broilers. Poult. Sci. 95:2427–2434.

Akhlaghi, A., M. J. Zamiri, A. Zare Shahneh, Y. Jafari Ahangari, A. NejatiJavaremi, G. Rahimi Mianji, M. R. Mollasalehi, H. Shojaie, A. A. Akhlaghi, H. Deldar, H. Atashi, Z. Ansari Pirsaraei, and M. Zhandi. 2012. Maternal hyperthyroidism is associated with a decreased incidence of cold-induced ascites in broiler chickens. Poult. Sci. 91:1165–1172.

Akhlaghi, A., M. J. Zamiri, Y. Jafari Ahangari, M. R. Mollasalehi, H. Shojaie, H. Atashi, B. Navidshad, A. A. Akhlaghi, and M. Dadpasand. 2013. Growth performance and intestinal morphology in broiler chickens produced from hyperthyroid breeder hens. Anim. Prod. Sci. 53:1046–1051.

Baghbanzadeh, A., and E. Decuypere. 2008. Ascites syndrome in broilers: Physiological and nutritional perspectives. Avian. Pathol. 37:117–126.

Balog, J. M., B. D. Kidd, W. E. Huff, G. R. Huff, N. C. Rath, and N. B. Anthony. 2003. Effect of cold stress on broilers selected for resistance or susceptibility to ascites syndrome. Poult. Sci. 82:1383–1387.

Bobek, S., M. Jastrzebski, and M. Pietras. 1977. Age-related changes in oxygen consumption and plasma thyroid hormone concentration in the young chicken. Gen. Comp. Endocrinol. 31:169–174.

Buys, N., J. Buyse, M. Hassanzadeh-Ladmakhi, and E. Decuypere. 1998. Intermittent lighting reduces the incidence of ascites in broilers: An interaction with protein content of feed on performance and the endocrine system. Poult. Sci. 77:54–61.

Currie, R. J. W. 1999. Ascites in poultry: Recent investigations. Avian Pathol. 28:313–326.

Ebrahimi, M. R., Y. JafariAhangari, M. J. Zamiri, A. Akhlaghi, and H. Atashi. 2012. Doespreincubational in ovo injection of buffers or antioxidants improve the quality and hatchability in long-term stored eggs? Poult. Sci. 91:2970–2976

Ernst, R. A., F. A. Bradley, U. K. Abbott, and R. M. Craig. 2004. Egg candling and breakout analysis for hatchery quality assurance and analysis of poor hatches. University of California, Division of Agriculture and Natural Resources.pub. 8134.

Geng, A. L., Y. M. Guo, and Y. Yang. 2004. Reduction of ascites mortality in broilers by coenzyme Q10. Poult. Sci. 83:1587–1593.

Gleeson, M. 1986. Respiratory adjustments of the unanaesthetized-chicken*Gallus domesticus*to elevated metabolism elicited by 2, 4-dinitrophenol or cold exposure. Comp. Biochem. Physiol. 83:283–289.

Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

Hulet, R., G. Gladys, D. Hill, R. Meijerhof, and T. El-Shiekh.2007. Influence of eggshell embryonic incubation temperature and broiler breeder flock age on posthatch growth and performance and carcass characteristics. Poult. Sci. 86:408–412.

Ipek, A., U. Sahan, S. C. Baycan, and A. Sozcu. 2014. The effects of different eggshell temperatures on embryonic development, hatchability, chick quality and first week broiler performance. Poult. Sci. 93:464–472.

Joseph, N. S., A. Lourens, and E. T. Moran, Jr. 2006. The effects of suboptimal eggshell temperature during incubation on broiler chickquality, live performance, and further processing yield. Poult. Sci. 85:932–938.

Julian, R. J. 1987. The effect of increased sodium in the drinking water on right ventricular hypertrophy, right ventricular failure and ascites in broiler chickens. Avian Pathol. 16:61–71.

Julian, R. J. 1993. Ascites in poultry. Avian Pathol. 22:419–454.

Kamely, M., M. A. KarimiTorshizi, and S. Rahimi. 2015. Incidence of ascites syndrome and related hematological response in short term feed-restricted broilers raised at low ambient temperature. Poult. Sci. 94:2247–2256.

Leksrisompong, N., H. Romero-Sanchez, P. W. Plumstead, K. E. Brannan, and J. Brake. 2007. Broiler incubation. 1. Effect of elevated temperature during late incubation on body weight and organs of chicks. Poult. Sci. 86:2685–2691.

Lourens, A., and J. H. Van Middelkoop. 2000. Embryo temperature affects hatchability and grow-outperformance of broilers. Avian Poult. Biol. Rev. 11:299–301.

Lourens, A., H. V. D. Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2007. Effects of eggshell temperature and oxygen concentration centration on embryo growth and metabolism during incubation. Poult. Sci. 86:2194–2199.

Lourens, A., H. V. D. Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and post-hatch development. Poult. Sci. 84:914–920.

Luger, D., D. Shinder, and S. Yahav. 2002. Hyper- or hypothyroidism: Its association with the development of ascites syndrome in fast growing chickens. Gen. Comp. Endocrinol. 127: 293–299.

Luger, D., D. Shinder, V. Rzepakovsky, M. Rusal, and S. Yahav. 2001. Association between weight gain, blood parameters, and thyroid hormones and the development of ascites syndrome in broiler chickens. Poult. Sci. 80:965–971.

Maatjens, C. M., I. A. M. Reijrink, R. Molenaar, C. W. Pol, B. Kemp, and H. van den Brand. 2014. Temperature and CO2 during the hatching phase. I. Effects on chick quality and organ development. Poult. Sci. 93:645–654.

Maxwell, M. H., G. W. Robertson, and S. Spence. 1986. Studies on ascites syndrome in young broilers. I. Haematology and pathology. Avian Pathol. 15:511–524.

McNabb, F. M. A. 2006. Avian thyroid development and adaptive plasticity. Gen. Comp. Endocrinol. 147:93–101.

McNabb, F. M. A. 2007. The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. Crit. Rev. Toxicol. 37:163–193.

Meijerhof, R. 2009. Incubation principles: What does the embryo expect from us?. In Proccedings of 20th Annual Australian Poultry Science Symposium, 9–11 February 2009, Sidney, Australia, pp. 106–111.

Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. V. D. Brand. 2011. High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. Poult. Sci. 90:624–632.

Noble, R. C. 1987. Lipid metabolism in the chick embryo: Some recent ideas. J. Exp. Zool. Suppl. 1:65–73.

Ocampo, L., U. Cortez, H. Sumano, and E. Avila. 1998. Use of low doses of clenbuterol to reduce incidence of ascites syndrome in broilers. Poult. Sci. 77:1297–1299.

Palo, P. E., J. L. Sell, F. J. Piquer, L. Vilaseea, and M. F. Soto Salanova. 1995. Effect of early nutrient restriction on broiler chickens performance and digestive enzyme activities. Poult. Sci. 74:1470–1483.

Pavlidis, H. O., J. M. Balog, L. K. Stamps, J. D. Hughes, W. E. Huff, and N. B. Anthony. 2007. Divergent selection for ascitesincidence in chickens. Poult. Sci. 86:2517–2529.

Puvadolpirod, S., and J. P. Thaxton. 2000. Model of physiological stress in chickens 3. Temporal patterns of response. Poult. Sci. 79:377–382.

Rajani, J., M. A. KarimiTorshizi, and S. Rahimi. 2011. Control of ascites mortality and improved performance and meat shelf-life in broilers using feed adjuncts with presumed antioxidant activity. Anim. Feed Sci. Tech. 170:239–245.

Romjin, C., and W. Lokhorst. 1955. Chemical heat regulation in the chick embryo. Poult. Sci. 34:649–654.

Santin, E., A. Maiorka, W. J. C. Polveiro, A. C. Paulillo Laurentiz, S. A. Borges, and A. V. Fischer da Silva. 2003. Effect of environmental temperature on immune response of broilers. J. Appl. Poult. Res. 12:247–250.

SAS Institute Inc. 2002. JMP User's Guide. Version 5. SAS Institute. Inc., Cary, NC.

Schalm, O. W., N. C. Jain, and E. J. Caroll. 1975. Veterinary Haematology, 3rd Ed. Pages 15–81, Lea and Febiger, Philadelphia.

Shahir, M. H., S. Dilmagani, and B. Tzschentke. 2012. Early-age cold conditioning of broilers: Effects of timing and temperature. Br. Poult. Sci. 53:538–544.

Shinder, D., D. Luger, M. Rusal, V. Rzepakovsky, V. Bresler, and S. Yahav. 2002. Early age cold conditioning in broiler chickens (Gallus domesticus): Thermo-tolerance and growth responses. J. Therm. Biol. 27:517–523.

Shinder, D., M. Rusal, M. Giloh, and S. Yahav. 2009. Effect of repetitive acute cold exposures during the last phase of broiler embryogenesison cold resistance through the life span. Poult. Sci. 88:636–646.

Shinder, D., M. Rusal, M. Giloh, S. Druyan, Y. Piestun, and S. Yahav. 2011. Improvement of cold resistance and performance of

broilers by acute cold exposure during late embryogenesis. Poult. Sci. 90:633–641.

Shlosberg, A., E. Berman, U. Bendheim, and I. Plavnik. 1991. Controlled early feed restriction as a potential means of reducing the incidence of ascites in broilers. Avian Dis. 35:681–684.

Shlosberg, A., M. Bellaiche, E. Berman, A. Ben David, N. Deeb, and A. Cahaner. 1998. Comparative effects of added sodium chloride, ammonium chloride, or potassium bicarbonate in the drinking water of broilers, and feed restriction, on the development of the ascites syndrome. Poult. Sci. 77:1287–1296.

Siegel, H. S., and J. W. Latimer. 1984. Interaction of high temperature and *Salmonella pullorum*antigenconcentration on serum agglutinin and corticosteroid responses in White Rock chickens. Poult. Sci. 63:2483–2491.

Solis de los Santos, F., M. B. Farnell, G. Tellez, J. M. Balog, N. B. Anthony, A. Torres-Rodriguez, S. Higgins, B. M. Hargis, and A. M. Donoghue. 2005. Effect of prebiotic on gut development and ascites incidence of broilers reared in a hypoxic environment. Poult. Sci. 84:1092–1100.

Tankson, J. D., J. P. Thaxton, and Y. Vizzier-Thaxton. 2002. Biochemical and immunological changes in chickens experiencing pulmonary hypertension syndrome caused by Enterococcus faecalis. Poult. Sci. 81:1826–1831.

Tona, K., F. Bamelis, B. De Ketelaere, V. Bruggeman, V. M. B. Moraes, J. Buyse, O. Onagbesan, and E. Decuypere. 2003. Effects of egg storage time on spread of hatch, chick quality and chick juvenile growth. Poult. Sci. 82:736–741.

Tona, K., O. Onagbesan, B. De Ketelaere, E. Decuypere, and V. Bruggeman. 2004. Effects of age of broiler breeders on egg quality, hatchability, chick quality, chick weight and chick post-hatch growth to 24 days. J. Appl. Poult. Res. 13:10–18.

Van der Geyten, S., I. Van den Eynde, I. B. Segers, E. R. Kuhn, and V. M. Darras. 2002. Differential expression of iodothyroninedeiodinases in chicken tissues during the last week of embryonic development. Gen. Comp. Endocrinol. 128:65–73.

Wasan, S. M., J. H. Sellin, and R. Vassilopoulou-Sellin. 2005. The gastrointestinal tract and liver in hypothyroidism. Pages 796–802 in Werner and Ingbar's The Thyroid: A Fundamental and Clinical Text. L. E. Braverman, and R. D. Utiger, ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Wideman, R. F. 2001. Pathophysiology of heart/lung disorders: Pulmonary hypertension syndrome in broiler chickens. Worlds Poult. Sci. J. 57:289–307.

Wideman, R. F. J., and H. French. 2000. Ascites resistance of progeny from broiler breeders selected for two generations using chronic unilateral pulmonary artery occlusion. Poult. Sci. 79:396–401.

Willemsen, H., N. Everaert, A. Witters, L. De Smit, M. Debonne, F. Verschuere, P. Garain, D. Berckmans, E. Decuypere, and V. Bruggeman. 2008. Critical assessment of chick quality measurements as an indicator of posthatch performance. Poult. Sci. 87:2358–2366.

Wilson, C. M., and F. M. A. McNabb. 1997. Maternal thyroid hormones in Japanese quail eggs and their influence on embryonic development. Gen. Comp. Endocrinol. 107:153–165.

Wittmann, J., W. Kugler, and H. Petry. 1983. Motility pattern and lung respiration of embryonic chicks under the influence of l-thyroxine and thiourea. Comp. Biochem. Physiol. A Comp. Physiol. 75:379–384.

Yahav, S., A. Straschnow, I. Plavnik, and S. Hurwitz. 1997. Blood system response of chickens to changes in environmental temperature. Poult. Sci. 76:627–633.

Yalçın, S., S. Özkan, P. Siegel, C. Yenisey, and M Akşit. 2012. Manipulation of incubation temperatures to increase cold resistance of broilers: Influence on embryo development, organ weights, hormones and body composition. Poult. Sci. 49:133–139

Zhai, W., L. W. Bennett, P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011a. Effects of in ovo injection of carbohydrates on somatic characteristics and liver nutrient profiles of broiler embryos and hatchlings. Poult. Sci. 90:2681–2688.

Zhai, W., P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011b. Effects of in ovo injection of carbohydrates on embryonic metabolism, hatchability, and subsequent somatic characteristics of broiler hatchlings. Poult. Sci. 90:2134–2143.