Effects of breeder age, broiler strain, and eggshell temperature on development and physiological status of embryos and hatchlings

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ABSTRACT Breeder age and broiler strain can influence the availability of nutrients and oxygen, particularly through differences in yolk size and shell conductance. We hypothesized that these egg characteristics might affect embryonic responses to changes in eggshell temperature (EST). This study aimed to investigate the effect of breeder age, broiler strain, and EST on development and physiological status of embryos. A study was designed as a 2 × 2 × 2 factorial arrangement using 4 batches of 1,116 hatching eggs of 2 flock ages at 29 to 30 wk (young) and 54 to 55 wk (old) of Ross 308 and Cobb 500. EST of 37.8 (normal) or 38.9°C (high) was applied from incubation d 7 (E7) until hatching. The results showed that breeder age rather than broiler strain had an influence on yolk size ($P = 0.043$). The shell conductance was higher in Ross 308 than in Cobb 500 ($P < 0.001$). A high EST resulted in a higher yolk free body mass (YFBM) compared to the normal EST at E14 and E16, but at 3 h after hatch YFBM was lower when eggs were incubated at high EST compared to normal EST (all $P < 0.001$). Cobb 500 eggs yielded embryos with a lower YFBM at E14, E18, and 3 h after hatch (all $P < 0.05$) than Ross 308 eggs. Breeder age had no effect on YFBM, but the RSY weight was higher in embryos from the old flock compared to the young flock embryos at E14 and E16 (both $P < 0.05$). A 3-way interaction among breeder age, strain, and EST was found, especially for incubation duration, navel quality, and relative heart and stomach weights at 3 h after hatch (all $P < 0.05$). Based on the results obtained, we conclude that oxygen availability rather than nutrient availability determines embryonic development, and the egg characteristics affected embryonic responses to changes of EST, especially for variables related to chick quality.

Key words: breeder age, broiler strain, eggshell temperature, embryonic development

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INTRODUCTION

During incubation, energy for embryonic development and growth is derived exclusively from egg nutrients. To metabolize nutrients, embryos uptake oxygen and use it in the biochemical process for energy production. The availability of both egg nutrients and oxygen therefore plays a crucial role in energy production for embryonic development and growth. From the start of incubation until incubation day 18 (E18), chicken embryos act poikilothermic, which means that their nutrient metabolism and thus embryonic growth during this period can be influenced by temperature (Tazawa et al. (1988). Studies in recent year have shown the importance of eggshell temperature (**EST**) and a constant EST of 37.8°C has been reported to be optimal for embryonic development and hatching success (Lourens et al., 2005; Lourens et al., 2007; Molenaar et al., 2010; 2011). An EST of 38.9°C from d 8 to d 19 of incubation (E8 to E19) initially increased embryonic development until the second wk of incubation, which was reflected in a high heat production (Lourens et al., 2007). However, at the last stage of incubation, a high EST had a negative impact on embryonic development. At the same stage of incubation, an increase of the oxygen level (25%) resulted in improved embryonic development (Lourens et al., 2007; Molenaar et al., 2010; 2011). These studies of EST and oxygen levels indicate that embryos, which initially have been accelerated in growth rate by a high EST, might at the end of incubation be unable to maintain nutrient metabolism at the level required to have a sufficient energy supply for development and growth. In other words, they might encounter an imbalance between requirements and availability of nutrients and oxygen and

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subsequently decreased nutrient metabolism and embryonic development.

Although egg nutrients are deposited in both the albumen and yolk, yolk nutrients function as the main nutritional supply providing approximately 90% of the total energy requirement (Romanoff, 1967; Noble and Cocchi, 1990). An alteration of yolk size is influenced mainly by breeder age (O'Sullivan et al., 1991; Hamidu et al., 2007; Nangsuay et al., 2011) and the amount of energy in the egg increases with yolk size (Nangsuay et al., 2013). Differences in broiler strains also have an influence on yolk size and consequently on the nutrient availability (Nangsuay et al., 2015). Yolk size does not only have an influence on the amount of yolk nutrients, but also on the capacity of the embryos to uptake yolk nutrients (Yadgary et al., 2013). Additionally, studies also have demonstrated that eggs from different breeder ages (O'Dea et al., 2004) and broiler strains (Nangsuay et al., 2015) tend to differ in eggshell conductance. As respiration gas exchanges occur across the eggshell, differences in eggshell conductance might affect the oxygen availability for the embryo, thereby affecting embryonic development and growth (Ar and Rahn, 1985). This means that hatching eggs originating from different breeder ages and strains could differ in oxygen and nutrient availability and this could affect nutrient metabolism and embryonic development during incubation. By integrating the available information from previous studies regarding the effects of EST and oxygen levels, it can be hypothesized that embryos from different breeder ages and broiler strains that differ in nutrient and oxygen availability, would react differently to an increase of EST. More specificly, it can be hypothesized that embryos from eggs with more nutrient availability and high eggshell conductance would cope better with an increased EST. Therefore, the objective of this study was to determine effects of breeder age, broiler strains, and EST on development and physiological status of embryos and hatchlings.

MATERIALS AND METHODS

Experimental Design

The experiment was designed as a 2 × 2 × 2 factorial arrangement with 2 breeder ages (young and old), 2 broiler strains (Ross 308 and Cobb 500), and 2 EST (37.8 and 38.9°C). In all treatments, EST was applied from E7 until the eggs were transferred to the hatching baskets (E18). Thereafter the machine temperature was fixed corresponding to an EST of 37.8 or 38.9°C. The experiment was performed in 4 consecutive batches and each treatment was repeated twice. Within each batch, eggs were obtained from the same breeder age of both strains and the eggs of each strain were incubated at 2 EST levels. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, the Netherlands.

Hatching Eggs and Egg Storage

Four batches of 1,116 good quality hatching eggs of 2 flock ages at 29 to 30 wk (young; **Y**) and 54 to 55 wk (old; **O**) of Ross 308 (**R**) and Cobb 500 fast feathering (**C**) were obtained from commercial broiler breeder farms. The broiler breeders of all flocks received commercially available diets. The diets for each group were as follows; YC = 14.9% CP, 5.2% crude fat, 2,775 kcal/kg ME, and 1.9% linoleic acid; YR = 14.7% CP, 4.8% crude fat, 2,813 kcal/kg ME, and 1.8% linoleic acid; OC = 14.6% CP, 4.8% crude fat, 2,772 kcal/kg ME, and 1.8% linoleic acid; OR = 14.2% CP, 5.3% crude fat, 2,767 kcal/kg ME, and 2.0% linoleic acid. Within each batch, eggs of both strains were obtained from the same breeder age. To assess normal average and variation of egg weight of each breeder flock, 150 eggs of each flock were weighed individually during the egg collection day. The average egg weight and standard variation of each strain at a given age were as follows: YR = 56.35 ± 3.77 g, OR = 68.75 ± 5.16 g, YC = 57.41 ± 4.10 g, and OC = 70.84 ± 5.52 g. Thereafter, for each batch, a total of 1,116 eggs (558 eggs of each strain) were selected at an egg weight range between 60 and 63 g. Per batch, 30 eggs of each strain and breeder age were used to determine egg composition. The remaining eggs of each strain were placed on 6 setter trays (88 eggs per tray) and were stored at 20°C and 55 to 60% relative humidity for 2 to 3 d.

Incubation

Incubation E0 to E7: Prior to the start of incubation, average egg weight of each tray was determined. All 12 setter trays (6 trays of each strain) of the 2 different strains were alternately placed in the same setter with a maximum setting capacity of 1,408 eggs (Hatchtech B.V., Veenendaal, the Netherlands). From E0 to E7, EST was maintained at 37.8°C and the relative humidity at 55 to 60%. The EST was measured by 4 sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK). The sensors were attached to 4 individual eggs, 2 eggs of each strain. The EST sensors were attached with a small piece of tape (Tesa BV, Almere, The Netherlands) in heat conducting paste (Schaffner Holding AG, Luterbach, Switzerland) to the eggshell at the equator of the egg. At E7, all eggs were candled and infertile and cracked eggs were removed from the trays. Thereafter weights of the fertile eggs plus tray and weight of the empty tray were measured. The average egg weight of the fertile eggs of each tray was used to calculate egg weight loss at E7—average egg weight of each tray at E0 minus the average egg weight of the fertile eggs of each tray at day E7. This egg weight loss was used in later calculations to determine eggshell conductance.

Incubation E7 to E18: After candling at E7, eggs of each strain were divided into a control and a treatment group and were incubated separately in one of 4

open-circuit climate respiration chambers (**CRC**). The CRC consisted of 2 CRC with a size of 267 L (small; Lourens et al., 2006) and 2 CRC with a size of 1,800 L (large; Verstegen and Henken, 1987). Each treatment was repeated twice, one replicate in a small CRC and the other replicate in a large CRC. Due to difference in CRC volume, approximately 70% of the eggs of each strain within each batch were incubated in the large CRC and the remaining eggs were incubated in the small CRC. All 4 CRC were equipped with the same system for egg turning, ventilation, temperature, and humidity control as described by Lourens et al. (2006). Each CRC has one Vaisala HMT330 series combi sensor (Vaisala Company, Vantaa, Finland) to measure machine temperature and relative humidity. The EST was measured by 5 sensors (Pt-100, Sensor Data BV, Rijswijk, The Netherlands) per CRC, which were attached to 5 individual fertile eggs in each CRC. The EST sensors were attached with tape as explained above. Between E7 to E18, EST of the control group was maintained at 37.8°C and at 38.9°C for the treatment group. The relative humidity was maintained at 55 to 60% for both the control and treatment groups. During incubation, EST from the 5 individual fertile eggs was measured continuously and the machine temperature was adjusted automatically every 5 min if the median EST differed from the target EST.

At E18, all eggs were candled and thereafter fertile eggs were transferred to hatching baskets. The machine temperature set point was set at a constant value that corresponded to an EST just before transfer of 37.8°C for the control group and 38.9°C for the treatment group. For the remaining time until hatching, machine temperature was fixed and EST was allowed to change.

Fresh Egg Composition

Fresh egg composition was measured to investigate nutrient availability in the eggs. In each batch, 30 eggs of each strain were boiled for 10 min and thereafter albumen and yolk were separated and weighed. The eggshell, excluding shell membranes, was dried for 24 h at room temperature and weighed. Albumen and yolk were stored at −20°C for further analyses.

Eggshell Conductance

Eggshell conductance influences diffusion rate of O_2 across the eggshell and thus oxygen availability for embryos. The eggshell conductance was calculated by using egg weight loss of fertile eggs between E0 and E7. Because the egg weight loss is a function of time, eggshell conductance and the water vapor pressure deficit (ΔP_{H2O}) across the egg shell, Meijerhof and van Beek (1993) demonstrated the eggshell conductance can be calculated by determination of egg weight loss under known $\Delta P_{H2O.}$ The water vapor pressure (P_{H2O}) is a function of temperature and relative humidity. To determine P_{H2O}, the average temperature and relative humidity between E0 and E7 was determined. The calculation for eggshell conductance was made as follows; egg weight loss / ΔP_{H2O}; where egg weight loss (mg/h) = egg weight loss of fertile eggs at E7/ (7 × 24) and ΔP_{H2O} (kPa) = average vapor pressure deficit between E0 to E7. To calculate $\Delta P_{H2O,}$ water vapor pressure inside the eggs was determined as the saturation vapor pressure at 37.8°C and 100% relative humidity. The water vapor pressure outside of the eggs was calculated based on average temperature (°C) and humidity (%) of the machine between E0 to E7.

Embryonic Development

To investigate an influence of breeder age, broiler strain, and EST on embryonic development, the measurements were made during incubation. At E7, 15 fertile eggs of each strain from each batch were randomly sampled. The eggs were opened and the embryo yolk free body was separated, dipped on tissue paper to dry, and weighed to obtain yolk free body mass **(YFBM)**. At E14, 30 eggs, and at E16, 15 eggs per treatment per batch were sampled. The eggs were opened and embryo YFBM and residual yolk (**RSY**) were separated and weighed. At E18, 22 to 25 fertile eggs of each treatment per batch were randomly sampled. Blood samples were withdrawn from the jugular vein of the embryos and thereafter embryo YFBM was determined. After decapitation, the liver was removed, weighed, and immediately stored in liquid nitrogen. The RSY and heart were removed and weighed. The YFBM of embryos at E18 was stored at −20°C for further analyses.

All other eggs that were transferred to hatching baskets were checked every 3 h for the hatching moment (chicks completely emerged from the eggshell) starting at 463 h after the start of incubation. The incubation duration was defined as the time between start of incubation and the hatching moment. At 3 h after hatch, chicks were taken out of the chambers and then cloacal temperature was immediately measured by using an infrared ThermoScan 4520 ExacTemp (Braun GmbH, Kronberg, Germany). The navel quality was assessed as 1 = good (nothing left outside the navel), 2 = moderate (a little black string or little black button smaller than 2 mm left outside the navel) or 3 = poor (a black button exceeding 2 mm left outside the navel). Thereafter, chick weight was measured and blood was collected after decapitation (0.5 to one mL). Chicks were opened and the liver was removed, weighed, and immediately stored in liquid nitrogen. The RSY and heart were removed and weighed. Chick YFBM was determined as chick weight minus RSY weight. Chick YFBM and RSY were stored at −20°C for further analyses. The stomach and intestines of the embryos at E18 and chicks at 3 h after hatch were weighed after defrosting the YFBM at room temperature for 24 h. All organ weights are expressed as percentage of YFBM. Hatch of fertile

was calculated as (number of hatched chick/number of fertile eggs that were transferred to hatching basket) × 100.

Plasma Metabolite and Hepatic Glycogen Determination

Plasma metabolite and hepatic glycogen are indications for physiological status of the embryos, which might be different due to variation of nutrients and oxygen availability. At E18, the sampled fertile eggs were opened and blood was withdrawn from the jugular vein using a 30-gauge needle and 1-mL syringe, flushed with 10% heparin. Blood was collected for approximately 0.3 to 0.5 mL per embryo in a 1.5 mL Eppendorfs containing 0.01 mL of 10% heparin. Blood was centrifuged at 12,000 g for one min at room temperature and plasma was stored at −20°C for further analyses. At 3 h after hatch, chicks were decapitated and blood was collected in a 4-mL blood tube containing 10 mg of sodium fluoride and 8 mg of potassium oxalate (BD Vacutainer, Franklin Lakes, NJ). An extra 0.02 mL of 10% heparin was added and mixed into the tube before sampling. Blood was centrifuged at 2,000 × g for 10 min at room temperature and plasma was stored at −20°C until further analyses. Plasma glucose, lactate, and uric acid concentrations of embryos at E18 and at 3 h after hatch were determined with commercially available enzymatic photometric kits (DiaSys Diagnostic Systems International, Holzheim, Germany).

Livers of embryos at E18 and chicks at 3 h after hatch were stored at −80°C until further analysis. The hepatic glycogen determination was performed as described by Molenaar et al. (2010). All procedures for hepatic glycogen determination were carried out on ice. The whole liver was homogenized with a glass stirring spoon after the addition of one μL of 7% $HCLO_4$/mg of wet tissue. The suspension was centrifuged (2,900 × *g*) at 4°C for 15 min. The supernatant was decanted, cleaned with one mL petroleum ether, and frozen at −80°C until further analysis. The supernatant was defrosted, centrifuged, and decanted again. Hepatic glycogen was determined by the iodine binding assay using an iodine (I_2-KI) solution containing saturated CaCl2 and the absorbance was measured by using microtitre plate reader at 450 nm. (Dreiling et al., 1987). Hepatic bovine glycogen (Type IX, Sigma-Aldrich Chemie GmbH, Steinheim,Germany) was used as a standard.

Statistical Analyses

The setter tray was used as the experimental unit for egg weight loss and eggshell conductance at E7. For other variables, egg and chick were used as the experimental unit in the statistical analyses. Distributions of means and residuals were examined to verify model assumptions. Lactate and uric acid at E18 were transformed to log10 and uric acid at 3 h after hatch was transformed to square-root data to obtain normal distribution. All data, except navel quality and hatch of fertile were analyzed using PROC MIXED in the statistical software package SAS 9.3 (SAS Institute Inc. 2002–2010, Cary, NC,). The model used for the statistical analyses of fresh egg composition, egg weight loss at E7, and eggshell conductance was

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + C(A_i) + e_{ijk} \quad (1)$$

where Y_{ijk} was the dependent variable, μ was the overall mean, A_i was the breeder age (i = young or old), B_j was the strain (j = Cobb 500 or Ross 308), AB_{ij} was the interaction between breeder age and strain, $C(A_i)$ was the breeder age nested within 4 batches and this term was used as an random effect, and e_{ijk} was the error term. Variables measured after E7, when the EST treatment was applied, were analyzed using PROC MIXED in the statistical software package SAS 9.3 (SAS Institute Inc. 2002–2010, Cary, NC) using model [1] extended with the EST (D_l; l = 37.8 or 38.9) and interactions of the other factors with EST (model 2). Type of climate respiration chambers (**CRC**; small and large) was initially included in model [2], but statistic results showed no influence of CRC on results obtained and thereafter this factor was excluded from the analysis. The navel quality scores were converted to a binary variable, where score 1 and score 2 were converted to 0 and score 3 was converted to 1. The statistical analyses for navel quality and hatch of fertile were performed using PROC GLIMMIX in the statistical software package SAS 9.3 (SAS Institute Inc. 2002–2010, Cary, NC) using model 2. Least square means were compared using Bonferroni adjustments for multiple comparisons. Values are expressed as LS means. For lactate and uric at E18 and uric acid at 3 h after hatch, LS means of original data are presented, combined with *P*-values of the transform data. In all cases, a difference was considered significant at $P \leq 0.05$.

RESULTS

Fresh Egg Composition, Egg Weight Loss, and Eggshell Conductance

A 2-way interaction between breeder age and strain was found for yolk weight ($P = 0.043$; Table 1). Yolk weight was higher in eggs of old breeder flocks than that of young breeder flocks in both strains, but the difference in Cobb 500 was smaller than in Ross 308 (3.20 and 3.81 g, respectively).

Although we selected on egg weight range, egg weight was higher in eggs from old breeder flocks than from young breeder flocks ($\Delta = 0.5$ g; $P = 0.048$). Egg weight of Cobb 500 and Ross 308 did not differ. Albumen weight was lower in eggs of old breeder flocks than that of young breeder flocks ($\Delta = 2.95$ g; $P = 0.003$), but did not differ between strains. Shell weight did not differ between breeder ages and strains. The

Table 1. Fresh egg, yolk, albumen, and shell weights (g), ratio of yolk to albumen, egg weight loss at incubation day (E) 7 (%), and eggshell conductance (mg/h/kPa) of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock (LSmeans).[1]

Effect	Egg, g	Yolk, g	Albumen, g	Shell, g	Yolk: Albumen	Egg weight loss, %[2]	Eggshell conductance, mg/h/kPa[2]
Breeder age × strain							
Y C	61.02	17.13[b]	38.39	5.50	0.45	3.05	4.04
Y R	61.19	16.89[b]	38.61	5.68	0.44	3.26	4.31
O C	61.56	20.33[a]	35.69	5.55	0.57	3.30	4.41
O R	61.64	20.70[a]	35.42	5.53	0.59	3.41	4.55
SEM	0.11	0.15	0.17	0.06	0.01	0.11	0.15
Breeder age (wk)							
Y	61.10[b]	17.01	38.50[a]	5.59	0.44[b]	3.16	4.18
O	61.60[a]	20.51	35.55[b]	5.54	0.58[a]	3.36	4.48
SEM	0.08	0.10	0.12	0.05	0.004	0.11	0.15
Strain							
C	61.29	18.73	37.04	5.52	0.51	3.18[b]	4.22[b]
R	61.41	18.79	37.02	5.60	0.51	3.33[a]	4.43[a]
SEM	0.08	0.10	0.13	0.04	0.004	0.08	0.11
Source of variation					*P*-values		
Breeder age × strain	0.724	0.043	0.152	0.079	0.071	0.141	0.137
Breeder age	0.048	0.002	0.003	0.508	0.002	0.329	0.287
Strain	0.273	0.639	0.887	0.164	0.697	<.0001	<.0001

[1]n = 60 for treatment combination of breeder age × strain.
[2]n = 12 for treatment combination of breeder age × strain.
[a,b]LSmeans lacking a common superscript within a column and factor differ ($P \leq 0.05$).

ratio of yolk to albumen was higher in eggs of old breeder flocks than that of young breeder flocks ($\Delta = 0.14$; $P = 0.002$), whereas the ratio of yolk to albumen did not differ between strains. At E7, egg weight loss ($\Delta = 0.15\%$; $P < 0.001$) and eggshell conductance ($\Delta = 0.21$ mg/h/kPa; $P < 0.001$) were higher in Ross 308 than in Cobb 500, but did not differ between breeder ages.

Yolk Free Body Mass (YFBM), Residual Yolk (RSY), Hatch of Fertile Egg (HOF), Incubation Duration, and Navel Quality

YFBM of embryos at E7, E14, E16, E18, and at 3 h after hatch and chick weight at 3 h after hatch did not differ between breeder ages (Table 2). YFBM of embryos at E7 and E16 did not differ between strains, but at E14 ($\Delta = 0.19$ g; $P < 0.026$), E18 ($\Delta = 0.59$ g; $P = 0.005$) and at 3 h after hatch ($\Delta = 0.59$ g; $P < 0.001$) YFBM was higher in Ross 308 embryos than in Cobb 500 embryos. At E14 ($\Delta = 1.11$ g; $P < 0.001$) and E16 ($\Delta = 0.94$ g; $P < 0.001$) the YFBM was higher in embryos at EST of 38.9° than at 37.8°C, but at 3 h after hatch the YFBM was lower ($\Delta = 0.83$ g; $P < 0.001$) in embryos at EST of 38.9 than 37.8°C. Chick weight at 3 h after hatch did not differ between strains and EST.

A 2-way interaction between breeder age and strain was found for RSY weight at 3 h after hatch ($P = 0.048$; Table 3). The RSY weight was lower in both young and old flocks of Ross 308 chicks than in Cobb 500 chicks, but this difference was more pronounced in the young flocks ($\Delta = 0.93$ g) than in the old flocks ($\Delta = 0.48$ g).

At E14 ($\Delta = 3.59$ g; $P = 0.002$) and E16 ($\Delta = 3.25$ g; $P = 0.028$) the RSY weight was higher in old flock embryos than in young flock embryos, but these effects disappeared at E18 and at 3 h after hatch (Table 3). At E14, E16, and at 3 h after hatch the RSY weight did not differ between strains, but at E18 the RSY weight was higher ($\Delta = 0.46$ g; $P = 0.021$) in Cobb 500 embryos than in Ross 308 embryos. At E14, E16, and E18, RSY weight did not differ between EST, but at 3 h after hatch, the RSY weight was 0.97 g higher in embryos incubated at an EST of 38.9 than 37.8°C ($P < 0.001$).

Percentage of hatch of fertile eggs did not differ among breeder ages, strains, and EST (Table 3).

A 3-way interaction among breeder age, strain, and EST was found for incubation duration ($P = 0.002$) and navel quality ($P = 0.009$) (Table 5). In young flocks, incubation duration in both EST was approximately 5 to 7 h shorter in Cobb 500 than in Ross 308. In old flocks, incubation duration at an EST of 37.8°C did not differ between strains; but at an EST of 38.9°C Cobb 500 chicks hatched approximately 9 h earlier than Ross 308 chicks. Navel quality expressed as percentage of chicks with navel score 3 (poor quality) was highest (35%) in Cobb 500 chicks at an EST at 38.9°C, whereas the other treatment groups were not different from each other.

Relative Organ Weights

A 3-way interaction between breeder age, strain, and EST was found for relative weights of heart ($P = 0.001$) and stomach ($P = 0.011$) 3 h after hatch (Table 5). At an EST of 37.8°C, relative heart weight did not differ between young and old flock chicks of either strain. An EST of 38.9°C led to a lower relative heart weight of chicks from both breeder ages and

Table 2. Yolk free body mass (YFBM, g) at E7, E14, E16, E18, and at hatch (3 h after hatch), chick weight at 3 h after hatch (g), and incubation duration (h) of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 or 38.9°C from E7 until hatching (LSmeans).[1]

	YFBM, g						
Effect[2]	E7[3]	E14	E16	E18	Hatch	Chick weight, g	Incubation duration, h
Breeder age × strain							
Y C	0.75	12.78	20.23	28.56	37.85	45.26	492
Y R	0.76	12.96	20.03	29.25	38.52	45.00	497
O C	0.79	12.93	19.82	28.29	37.74	45.38	488
O R	0.78	13.15	20.11	28.79	38.25	45.38	493
SEM	0.01	0.23	0.17	0.30	0.13	0.14	0.93
Breeder age × EST							
Y 37.8		12.34	19.61	28.77	38.71	45.17	499
Y 38.9		13.40	20.65	29.04	37.67	45.09	490
O 37.8		12.46	19.54	28.47	38.31	45.21	496
O 38.9		13.63	20.38	28.61	37.69	45.54	485
SEM		0.19	0.17	0.30	0.13	0.14	0.93
Strain × EST							
C 37.8		12.34	19.57	28.17	38.27	45.32	496
C 38.9		13.38	20.48	28.68	37.33	45.32	483
R 37.8		12.46	19.58	29.07	38.74	45.06	499
R 38.9		13.64	20.55	28.97	38.03	45.32	491
SEM		0.17	0.17	0.26	0.13	0.13	0.78
Breeder age							
Y	0.76	12.87	20.13	28.91	38.19	45.13	494
O	0.78	13.04	19.96	28.54	37.99	45.38	490
SEM	0.01	0.22	0.12	0.26	0.09	0.11	0.83
Strain							
C	0.77	12.86[b]	20.02	28.43[b]	37.80[b]	45.32	490
R	0.77	13.05[a]	20.07	29.02[a]	38.39[a]	45.19	495
SEM	0.01	0.16	0.12	0.21	0.09	0.10	0.65
EST							
37.8		12.40[b]	19.58[b]	28.62	38.51[a]	45.19	498
38.9		13.51[a]	20.52[a]	28.82	37.68[b]	45.32	487
SEM		0.16	0.12	0.21	0.09	0.10	0.65
Source of variation					*P*-values		
Breeder age × strain	0.333	0.790	0.160	0.671	0.535	0.264	0.302
Breeder age × EST		0.542	0.574	0.776	0.110	0.077	0.112
Strain × EST		0.433	0.866	0.154	0.370	0.274	<.001
Breeder age	0.187	0.627	0.448	0.420	0.276	0.248	0.073
Strain	0.933	0.026	0.802	0.005	<.001	0.272	<.001
EST		<.001	<.001	0.340	<.001	0.272	<.001

[1]n for each treatment combination of breeder age × strain, breeder age × EST and strain × EST at E14 = 120, E16 = 60, E18 = 88-100. n at hatch for chick weight and incubation duration = 120 to 132.

[2]Significant interaction between breeder age × strain × EST was found only for incubation duration and the results are presented in Table 5.

[3]n = 30 for treatment combination of breeder age × strain.

[a,b]LSmeans lacking a common superscript within a column and factor differ ($P \leq 0.05$).

strains and this effect was more pronounced in old Cobb 500 ($\Delta = 0.21\%$) than in the other breeder age x strain combinations ($\Delta = 0.11$ to 0.19%). Relative stomach weight of all groups did not differ between EST, but there were differences within each EST. At an EST of 37.8°C, relative stomach weight was higher in old Cobb 500 chicks than old Ross 308 chicks ($\Delta = 0.41\%$), whereas at an EST of 38.9°C, relative stomach weight was lower in young Ross 308 chicks than young Cobb 500 chicks ($\Delta = 0.30\%$).

In embryos at E18, a 2-way interaction between breeder age and EST was found for relative weight of liver ($P = 0.012$) and heart ($P < 0.001$) (Table 4). At an EST of 37.8°C, relative liver weight of embryos at E18 did not differ between breeder ages, whereas at this moment in incubation an EST at 38.9°C led to a lower relative liver weight only in the young flock ($\Delta = 0.15\%$). At an EST of 37.8°C, relative heart weight of embryos at E18 did not differ between breeder ages, whereas an EST at 38.9°C led to a lower relative heart weight in both young ($\Delta = 0.09\%$) and old flocks ($\Delta = 0.18\%$). A 2-way interaction between strain and EST was found for relative heart weight of embryos at E18 ($P = 0.048$) and relative intestinal weight of chicks at 3 h after hatch ($P = 0.032$). At an EST of 37.8°C, relative heart weight of embryos at E18 did not differ between strains, whereas an EST at 38.9°C led to a lower relative heart weight in both Cobb 500 ($\Delta = 0.16\%$) and Ross 308 ($\Delta = 0.12\%$). At an EST of 37.8°C, relative intestinal weight of chicks at 3 h after hatch did not differ, whereas at an EST of 38.9°C Ross 308 chicks had a higher relative intestinal weight than Cobb 500 chicks ($\Delta = 0.35\%$).

Relative stomach weight at E18 was higher in embryos from an EST of 38.9 than 37.8°C ($\Delta = 0.19\%$; $P = 0.002$; Table 4). Relative intestinal weight at E18

Table 3. Residual yolk (RSY) at E14, E16, E18, and at hatch (3 h after hatch, g), navel quality (% of navel score 3), and hatch of fertile eggs (HOF, %) of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) breeder eggs incubated at an eggshell temperature (EST) of 37.8 or 38.9°C from E7 until hatching (LSmeans).[1]

	RSY, g					
Effect[4]	E14	E16	E18	Hatch	Navel quality, %[2]	HOF, %[3]
Breeder age × strain						
Y C	14.17	13.29	12.32	7.40[a,b]	15.9	93.18
Y R	13.92	13.91	11.72	6.47[c]	9.6	94.56
O C	17.58	16.64	13.48	7.63[a]	19.1	95.00
O R	17.68	17.06	13.15	7.15[b]	6.3	96.67
SEM	0.14	0.44	0.37	0.11		
Breeder age × EST						
Y 37.8	14.19	13.73	11.99	6.46	7.3	94.78
Y 38.9	13.89	13.48	12.05	7.41	16.3	93.59
O 37.8	17.69	17.09	13.48	6.90	5.2	96.67
O 38.9	17.57	16.61	13.14	7.89	20.7	95.00
SEM	0.14	0.44	0.37	0.11		
Strain × EST						
C 37.8	15.99	15.29	13.04	7.04	7.3	96.85
C 38.9	15.76	14.64	12.75	7.99	24.4	92.36
R 37.8	15.89	15.52	12.43	6.32	5.1	94.49
R 38.9	15.71	15.45	12.44	7.31	10.6	95.47
SEM	0.14	0.36	0.30	0.11		
Breeder age						
Y	14.04[b]	13.60[b]	12.02	6.94	12.9	93.85
O	17.63[a]	16.85[a]	13.31	7.39	12.8	95.83
SEM	0.09	0.39	0.34	0.08		
Strain						
C	15.87	14.96	12.89[a]	7.52	17.2	93.69
R	15.80	15.49	12.43[b]	6.81	8.2	95.17
SEM	0.09	0.31	0.26	0.08		
EST						
37.8	15.94	15.41	12.74	6.68[b]	6.3	95.67
38.9	15.73	15.04	12.60	7.65[a]	17.9	93.88
SEM	0.09	0.31	0.26	0.08		
Source of variation				*P*-values		
Breeder age × strain	0.211	0.707	0.505	0.048	0.912	0.476
Breeder age × EST	0.548	0.678	0.308	0.883	0.810	0.920
Strain × EST	0.856	0.278	0.444	0.819	0.138	0.179
Breeder age	0.002	0.028	0.114	0.054	0.716	0.510
Strain	0.589	0.057	0.021	<.0001	0.029	0.863
EST	0.136	0.175	0.494	<.001	0.001	0.432

[1]n for each treatment combination of breeder age × strain, breeder age × EST and strain × EST at E14 = 120, E16 = 60, E18 = 88 to 100. n at hatch for chick weight, navel quality, and HOF = 120 to 132.
[2]The values are the percentage of chicks with navel score 3 (poor).
[3]The values are means of percentage of hatch of fertile eggs.
[4]Significant interaction between breeder age × strain × EST was found only for navel quality and the results are presented in Table 5.
[a–c]LSmeans lacking a common superscript within a column and factor differ ($P \leq 0.05$).

was higher in embryos from an EST of 38.9 than 37.8°C ($\Delta = 0.24\%$; $P < 0.001$). Relative stomach and intestinal weights of embryos at E18 did not differ between breeder ages and strains. Relative liver weight of chicks at 3 h after hatch was higher at an EST of 37.8 than at 38.9°C ($\Delta = 0.11\%$; $P < 0.001$). Breeder age and strain had no effect on relative liver weight of chicks at 3 h after hatch. Breeder age had no effect on relative intestinal weight of chicks at 3 h after hatch.

Total Hepatic Glycogen and Blood Metabolites

A 3-way interaction among breeder age, strain, and EST was found for total hepatic glycogen of embryos at E18 ($P = 0.029$), plasma glucose concentration of embryos at E18 ($P = 0.005$), and plasma glucose concentration of chicks at 3 h after hatch ($P = 0.023$) (Table 8). Total hepatic glycogen at E18 was higher in young Cobb 500 embryos at an EST of 37.8°C than in old Ross 308 embryos at an EST of 38.9°C ($\Delta = 11.73$ mg), whereas other groups were intermediate and not different from each other. At an EST of 37.8°C, plasma glucose concentration of embryos at E18 did not differ between young and old flocks of either strain, whereas an EST at 38.9°C led to a lower glucose concentration only in young Ross 308 embryos ($\Delta = 1.43$ mmol/L). At an EST of 37.8°C, plasma glucose concentration of chicks at 3 h after hatch was higher in old Cobb 500 than other groups, which were not different from each other. An EST at 38.9°C led to a

Table 4. Relative weights of liver, heart, stomach, and intestines (% of YFBM) of the embryos at E18 and chicks at 3 h after hatch of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 or 38.9°C from E7 until hatching (LSmeans).[1]

	Relative weight at E18, %				Relative weight at 3 h after hatch,%			
Effect[2]	Liver	Heart	Stomach	Intestines	Liver	Heart	Stomach	Intestines
Breeder age × strain								
Y C	1.94	0.60	4.18	1.69	2.19	0.81	5.60	3.63
Y R	1.93	0.62	4.09	1.64	2.23	0.83	5.42	3.87
O C	1.96	0.75	4.07	1.85	2.31	0.81	5.68	3.64
O R	1.97	0.75	4.08	1.97	2.33	0.84	5.41	3.88
SEM	0.03	0.07	0.07	0.10	0.03	0.01	0.05	0.09
Breeder age × EST								
Y 37.8	2.01^{a}	0.66^{a,b}	4.01	1.54	2.27	0.91	5.59	3.74
Y 38.9	1.86^{b}	0.57^{c}	4.25	1.80	2.16	0.74	5.43	3.77
O 37.8	1.98^{a}	0.84^{a}	4.00	1.79	2.37	0.91	5.61	3.77
O 38.9	1.94^{a,b}	0.66^{b,c}	4.15	2.00	2.26	0.74	5.49	3.76
SEM	0.03	0.07	0.07	0.10	0.03	0.01	0.05	0.09
Strain × EST								
C 37.8	2.01	0.76^{a}	4.03	1.63	2.32	0.90	5.72	3.69^{b,c}
C 38.9	1.90	0.60^{b}	4.22	1.92	2.19	0.73	5.57	3.59^{c}
R 37.8	1.98	0.75^{a}	3.98	1.70	2.32	0.91	5.48	3.81^{a,b}
R 38.9	1.91	0.63^{b}	4.18	1.88	2.23	0.76	5.35	3.94^{a}
SEM	0.03	0.05	0.07	0.07	0.02	0.01	0.05	0.08
Breeder age								
Y	1.97	0.61	4.13	1.67	2.21	0.82	5.51	3.75
O	1.94	0.75	4.07	1.90	2.32	0.82.82	5.55	3.76
SEM	0.02	0.07	0.06	0.09	0.02	0.01	0.03	0.09
Strain								
C	1.95	0.68	4.12	1.77	2.25	0.81	5.64	3.64
R	1.95	0.69	4.08	1.79	2.28	0.83	5.41	3.88
SEM	0.02	0.05	0.05	0.07	0.02	0.01	0.03	0.07
EST								
37.8	1.99	0.75	4.01^{b}	1.66^{b}	2.32^{a}	0.91	5.59	3.75
38.9	1.91	0.61	4.20^{a}	1.90^{a}	2.21^{b}	0.74	5.46	3.76
SEM	0.02	0.05	0.05	0.07	0.02	0.01	0.03	0.07
Source of variation				*P*-values				
Breeder age × strain	0.808	0.485	0.419	0.099	0.748	0.930	0.352	0.996
Breeder age × EST	0.012	<.001	0.407	0.546	0.887	0.763	0.679	0.724
Strain × EST	0.355	0.048	0.923	0.165	0.305	0.334	0.870	0.032
Breeder age	0.489	0.287	0.563	0.227	0.091	0.988	0.514	0.938
Strain	0.798	0.255	0.538	0.692	0.131	0.029	<.001	<.001
EST	<.001	<.001	0.002	<.001	<.001	<.001	0.004	0.788

[1]n for each treatment combination of breeder age × strain, breeder age × EST and strain × EST at E18 = 88 to 100, and at 3 h after hatch = 120 to 132.

[2]Significant interaction between breeder age × strain × EST was found for relative heart and stomach weight at 3 h after hatch and the results are presented in Table 5.

[a–c]LSmeans lacking a common superscript within a column and factor differ ($P \leq 0.05$).

lower glucose concentration only in old Cobb 500 chicks ($\Delta = 0.68$ mmol/L).

A 2-way interaction between breeder age and strain was found for uric acid concentration in plasma of chicks at 3 h after hatch ($P = 0.031$; Table 7). Uric acid in plasma of Cobb 500 chicks did not differ between breeder ages, whereas old Ross 308 chicks had a lower uric acid concentration than young Ross 308 chicks ($\Delta = 0.05$ mmol/L).

Total hepatic glycogen of chicks at 3 h after hatch did not differ between breeder ages, strains, and EST (Table 6). Lactate in plasma of embryos at E18 ($\Delta = 0.23$ mmol/L; $P = 0.003$) and chicks at 3 h after hatch ($\Delta = 0.31$ mmol/L; $P = 0.013$) was higher in Cobb 500 than in Ross 308 (Table 7). Lactate in plasma of embryos at E18 did not differ between breeder ages and EST. Lactate in plasma of chicks at 3 h after hatch was higher in an EST at 37.8 than 38.9°C ($\Delta = 0.36$ mmol/L; $P = 0.004$). Lactate of chicks at 3 h after hatch did not differ between breeder ages. Uric acid in plasma of embryos at E18 did not differ among breeder age, strains, and EST, and of chicks at 3 h after hatch did not differ between EST.

DISCUSSION

Chicken embryos uptake nutrients from albumen and yolk and these nutrients are oxidized to provide energy for development and growth. Although both albumen and yolk are used for energy production, egg yolk functions as the main nutritional supply accounting for approximately 90% of total energy requirement (Romanoff, 1967). An alteration of yolk size, due to changes in breeder age (Nangsuay et al., 2013) or strain (Nangsuay et al., 2015) has been shown to noticeably differentiate nutrient availability in the eggs, which might have

Table 5. Incubation duration (h), navel quality of chicks at 3 h after hatch (% of chicks with score 3), heart, and stomach weight (% YFBM) at 3 h after hatch of Cobb 500 (C) and Ross 308 (R) from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 and 38.9°C (LSmeans).[1]

Effect	Incubation duration, h	Navel quality, % of score 3[2]	Heart, % YFBM	Stomach, % YFBM
Breeder age × strain × EST				
Y C 37.8	497^{b}	10.80^{a,b}	0.89^{a}	5.62^{a,b}
Y C 38.9	486^{d}	18.90^{a,b}	0.74^{b,c}	5.58^{a,b}
Y R 37.8	502^{a}	3.40^{b}	0.93^{a}	5.56$^{a–c}$
Y R 38.9	493^{c}	13.40^{a,b}	0.74^{b,c}	5.28^{c}
O C 37.8	496^{b,c}	3.40^{b}	0.92^{a}	5.81^{a}
O C 38.9	480^{e}	35.10^{a}	0.71^{c}	5.56$^{a–c}$
O R 37.8	496^{b,c}	6.90^{b}	0.89^{a}	5.40^{b,c}
O R 38.9	489^{d}	5.60^{b}	0.78^{b}	5.42^{b,c}
SEM	1.10		0.02	0.07
Source of variation		*P*-values		
Breeder age × strain × EST	0.002	0.009	0.001	0.011

[1]n for incubation duration, navel quality, heart, and stomach weight at 3 h after hatch = 60 to 66.
[2]The values are the percentage of chicks with navel score 3 (poor).
[a–e]LSmeans lacking a common superscript within a column differ ($P \leq 0.05$).

an influence on embryonic development and nutrient metabolism during incubation. In the current study an attempt was made to confirm and extend previous findings regarding the composition of young and old flock eggs of Cobb 500 and Ross 308 strains. The average weight of the selected eggs in the current study was 0.5 g higher in old flocks than in young flocks, but we do not expect this relatively small difference to have a biological effect on results obtained.

A significant interaction between breeder age and strain for yolk weight indicated a pronounced effect of breeder age. Yolk weight was approximately 3.2 and 3.8 g higher in the old flocks of Cobb 500 and Ross 308, respectively, than in the young flocks of both strains. On the other hand, yolk weight did not differ for eggs of the same breeder age originating from different strains. As a consequence of changes of yolk weight with breeder age, ratio of yolk to albumen was higher in old flock eggs than young flock eggs. The current results confirm previous studies that with an increase in breeder age, the yolk size increases as well (Ahn et al., 1997; Nangsuay et al., 2011, 2013). In contrast to Nangsuay et al. (2015), Cobb 500 and Ross 308 did not differ in yolk weight. The inconsistency among studies for egg compositions and especially for yolk weight of Cobb 500 and Ross 308 could be due to the difference in distribution of the selected eggs compared to the flock average egg weight of each breeder age, which might have influenced the yolk-to-albumen ratio (Nangsuay et al., 2011). As the eggs in this experiment were selected on a specific weight range for both age groups and strains, weights of selected eggs were not fully representative for the normal egg weight distribution of the flocks. Another effect on yolk-to-albumen ratio of eggs might have been the energy intake of the breeders, which can be influenced by diet composition and feed quantity (Peebles et al., 2000). As the eggs were obtained from different commercial flocks using different diet compositions, an influence of diet and energy intake on the yolk size and yolk composition and therefore on nutrient availability cannot be totally excluded. Although both these factors might have slightly influenced the results, the current study suggests that due to alteration of yolk size the breeder age rather than strain influences nutrient availability in hatching eggs.

Embryonic Development and Hatchling

During incubation, the developing embryos require nutrients and oxygen for development and growth. The requirements for nutrients and oxygen are progressively increased with embryonic growth rate. Tazawa et al. (1988) demonstrated that embryos before E18 act as poikilothermic, which means that embryonic development and growth during this period are temperature dependent. In agreement with this observation, the current results showed that embryos at an EST of 38.9°C had a higher YFBM at E14 and E16 than that at an EST of 37.8°C. However, these effects disappeared at later stages of incubation and at 3 h after hatch chick YFBM was lower at a high EST than at a normal EST. These embryonic growth patterns manifest embryonic responses to EST, which might be associated with the propositions made by Tazawa et al. (1988) and Whittow and Tazawa. (1991). According to these authors, a higher embryonic growth at E14 and E16 might be explained by the temperature-dependent Arrhenius-limited stage, whereas a lower growth rate at a later stage could be explained by an oxygen-conductance-limited stage. As the Arrhenius-limited stage is characterized by a metabolic rate related to changes in temperature, a high EST at 38.9°C will have accelerated nutrient metabolism, thereby enhancing embryonic growth rate. Consequently, the demand for energy for maintenance of existing embryonic tissues and the continuing synthesis of new body tissues are progressively increased. To meet the embryonic demands

Table 6. Total hepatic glycogen at E18 and at 3 h after hatch (mg) of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 or 38.9°C from E7 until hatching (LSmeans).[1]

	Total hepatic glycogen, mg	
Effect[2]	E18	At 3 h after hatch
Breeder age × strain		
Y C	20.42	20.60
Y R	17.55	17.65
O C	18.20	24.14
O R	16.28	23.65
SEM	2.88	2.15
Breeder age × EST		
Y 37.8	20.97	18.91
Y 38.9	16.99	19.34
O 37.8	19.49	23.60
O 38.9	14.98	24.20
SEM	2.83	2.15
Strain × EST		
C 37.8	20.57	22.08
C 38.9	18.04	22.67
R 37.8	19.89	20.42
R 38.9	13.93	20.87
SEM	2.27	1.74
Breeder age		
Y	18.98	19.12
O	17.23	23.89
SEM	2.62	1.97
Strain		
C	19.31	22.37
R	16.91	20.65
SEM	2.00	1.52
EST		
37.8	20.23	21.25
38.9	15.99	21.77
SEM	2.00	1.52
Source of variation	*P*-values	
Breeder age × strain	0.754	0.308
Breeder age × EST	0.859	0.667
Strain × EST	0.262	0.953
Breeder age	0.684	0.229
Strain	0.118	0.153
EST	0.006	0.667

[1]n for each treatment combination of breeder age × strain, breeder age × EST, and strain × EST for total hepatic glycogen at E18 and at 3 h after hatch = 40.

[2]Significant interaction between breeder age × strain × EST was found for total hepatic glycogen at E18 and the results are presented in Table 8.

in this stage of development, nutrient and oxygen availability should be increased as well. A similar fresh yolk weight suggests that embryos of both EST had an equal nutrient availability at the start of incubation. However, acceleration of YFBM at E14 and E16 by an increase in EST implied a necessity for a higher nutrient metabolism to sustain growth. It can be expected that an accelerated growth rate at high EST resulting in an increased oxygen requirement has resulted in embryos reaching an oxygen-conductance-limited stage. Although sufficient nutrients are available, yolk utilization will be decreased due to limited oxygen availability on tissue level and subsequently yolk uptake will be affected. A higher RSY of chicks from high EST compared to normal EST could be a reflection of limited oxygen availability to metabolize yolk nutrients.

Oxygen uptake by embryos is influenced by eggshell conductance. The eggshell conductance determines the diffusion rate of oxygen and this characteristic is fixed once the shell is formed (Ackerman and Rahn, 1981). Ar and Rahn (1985) demonstrated that eggshell water vapor conductance and oxygen consumption rate at the pre-internal pipping stage are directly proportional to absolute mean growth rate of embryos (g per day). In agreement with a previous study (Nangsuay et al., 2015), the current results show that Cobb 500 eggs, which had a lower eggshell conductance than Ross 308 eggs, yielded a lower YFBM at E14, E18, and at 3 h after hatch. Since the embryos of both strains were equal in yolk size, which indicates similar nutrient availability at the start of incubation, it might be possible that oxygen limitation is a reason for the lower YFBM. Cobb 500 embryos might experience an imbalance between requirement and availability of oxygen already at E14. This condition might be continued until the moment of external pipping and as a result nutrient uptake from the yolk in Cobb 500 embryos will be lower than in Ross 308 embryos. Comparable observations were made in a study in chicks 3 d after hatch by Pulikanti et al. (2012). These authors reported that a specific eggshell water vapor conductance (eggshell water vapor conductance adjusted to 100 g of set egg weight basis) was positively correlated with chick carcass weight relative to egg weight, but was negatively correlated with yolk sac weight as a percentage of chick weight. Furthermore, Tona et al. (2010) reported that at the internal pipping stage Cobb eggs had a lower partial oxygen pressure (pO_2) in the air cell than Ross eggs, suggesting that Cobb embryos were experiencing limited oxygen availability, and consequently yolk nutrient utilization and yolk uptake might be affected. A higher RSY of embryos at E18 and chicks at 3 h after hatch in Cobb 500 compared to Ross 308 for both age groups might indicate a condition in which oxygen availability was limited for yolk nutrient metabolism. It could be implied that due to a lower eggshell conductance an earlier and probably a higher magnitude of oxygen-conductance-limited stage is induced in Cobb 500 compared to Ross 308. Consequently less oxygen is available for nutrient metabolism and as a result embryonic growth rate was decreased. Although both nutrient and oxygen availability are essential factors, the current results suggest that oxygen rather than nutrient level was limited for energy production to sustain embryonic growth. The current observation is supported by studies of Lourens et al. (2007) and Molenaar et al. (2010) who reported an increase of YFBM and a decrease of RSY of embryos subjected to a high oxygen level (25%) between E7 and E19.

The results obtained from eggs varying in yolk size, which suggested differences in nutrient availability due to breeder age, confirm the above suggestion. Eggs obtained from young and old flocks did not differ in eggshell conductance, which suggests equal oxygen availability for embryos of both breeder ages. The

Table 7. Blood plasma glucose, lactate, and uric acid (mmol/L) at E18 and at 3 h after hatch of Cobb 500 (C) and Ross 308 (R) eggs from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 or 38.9°C from E7 until hatching (LSmeans).[1]

	E18, mmol/L			At 3 h after hatch, mmol/L		
Effect[2]	Glucose	Lactate	Uric acid	Glucose	Lactate	Uric acid
Breeder age × strain						
Y C	9.82	1.65	0.15	11.12	2.86	0.12[a,b]
Y R	9.00	1.50	0.16	10.82	2.62	0.14[a]
O C	10.02	2.16	0.14	11.70	2.88	0.11[a,b]
O R	9.54	1.87	0.13	11.53	2.50	0.09[b]
SEM	0.57	0.34	0.01	0.19	0.15	0.01
Breeder age × EST						
Y 37.8	9.93	1.62	0.16	11.22	2.92	0.13
Y 38.9	8.89	1.53	0.15	10.72	2.55	0.13
O 37.8	10.03	2.00	0.13	11.80	2.86	0.10
O 38.9	9.53	2.03	0.14	11.42	2.51	0.11
SEM	0.57	0.34	0.01	0.19	0.15	0.01
Strain × EST						
C 37.8	10.31	1.99	0.14	11.66	2.95	0.12
C 38.9	9.53	1.82	0.15	11.16	2.78	0.11
R 37.8	9.65	1.63	0.14	11.36	2.83	0.12
R 38.9	8.89	1.74	0.14	10.99	2.29	0.12
SEM	0.42	0.25	0.01	0.15	0.14	0.01
Breeder age						
Y	9.41	1.58	0.15	10.97	2.74	0.13
O	9.78	2.02	0.13	11.61	2.69	0.10
SEM	0.56	0.34	0.01	0.17	0.12	0.01
Strain						
C	9.92	1.91[a]	0.15	11.41	2.87[a]	0.12
R	9.27	1.68[b]	0.14	11.17	2.56[b]	0.12
SEM	0.41	0.24	0.01	0.13	0.10	0.01
EST						
37.8	9.98	1.81	0.14	11.51	2.89[a]	0.12
38.9	9.21	1.78	0.14	11.07	2.53[b]	0.12
SEM	0.41	0.24	0.01	0.13	0.10	0.01
Source of variation			*P*-values			
Breeder age × strain	0.255	0.638	0.646	0.539	0.582	0.031
Breeder age × EST	0.059	0.205	0.449	0.584	0.940	0.499
Strain × EST	0.959	0.067	0.266	0.533	0.131	0.239
Breeder age	0.726	0.501	0.176	0.120	0.791	0.262
Strain	<.0001	0.003	0.729	0.027	0.013	0.671
EST	<.0001	0.817	0.961	<.001	0.004	0.795

[1]n for each treatment combination of breeder age × strain, breeder age × EST, and strain × EST for glucose, lactate and uric acid at E18 and at 3 h after hatch = 40.

[2]Significant interaction between breeder age × strain × EST was found for glucose at E18 and glucose at 3 h after hatch and the results are presented in Table 8.

[a,b]LSmeans lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 8. Total hepatic glycogen (mg) at E18, glucose at E18 and at 3 h after hatch (mmol/L) of Cobb 500 (C) and Ross 308 (R) from young (Y; 29 to 30 wk) and old (O; 54 to 55 wk) flock incubated at an eggshell temperature (EST) of 37.8 and 38.9°C (LSmeans).[1]

Effect	Total hepatic glycogen at E18, Mg	Glucose E18, mmol/L	Glucose at 3 h after hatch, mmol/L
Breeder age × strain × EST			
Y C 37.8	23.22[a]	10.14[a,b]	11.28[b]
Y C 38.9	17.62[a,b]	9.50[b]	10.96[b,c]
Y R 37.8	18.72[a,b]	9.72[a,b]	11.15[b,c]
Y R 38.9	16.37[a,b]	8.29[c]	10.48[c]
O C 37.8	17.92[a,b]	10.48[a]	12.04[a]
O C 38.9	18.47[a,b]	9.57[a,b]	11.36[b]
O R 37.8	21.07[a,b]	9.58[a,b]	11.56[a,b]
O R 38.9	11.49[b]	8.29[b]	11.49[a,b]
SEM	3.22	0.62	0.26
Source of variation		*P*-values	
Breeder age × strain × EST	0.029	0.005	0.023

[1]n for total hepatic glycogen at E18, glucose at E18, and glucose at 3 h after hatch = 20.

[a–c]LSmeans lacking a common superscript within a column differ ($P \leq 0.05$).

YFBM of embryos and chicks originating from young and old flock eggs, which varied in yolk size, were comparable at all measurement d. Although yolk utilization, reflected in RSY at 3 h after hatch, was higher in old flocks than in young flocks, the YFBM at hatch did not differ between either flock age. The current results disagree with the previous suggestions regarding yolk utilization as a determinant factor for embryonic growth (Byerly, 1932; Wilson, 1991). Similar results were demonstrated by Nangsuay et al. (2011) who reported a higher yolk utilization of embryos from old flocks than of young flocks, but the differences in yolk utilization had no effect on YFBM at hatch.

Differences in embryonic development in response to high EST also were shown in the internal organ weights relative to YFBM. These findings might be related to differentiation of internal organ growth in relation to embryonic developmental stages and nutrient metabolism, which in turn are related to availability of nutrients and oxygen. During incubation, development patterns of internal organs relative to embryonic weight differ; for example, the heart reaches maximum weight relative to embryonic weight at E5, whereas the liver, intestine, and gizzard reach this at E21 (Romanoff, 1960). In a condition in which there is an alteration of nutrient metabolism by a high EST and the trajectory of embryonic growth is changed, development of demand (e.g., skeleton and muscle) and supply organs (e.g. heart and liver) might subsequently be affected. The effects could be different for embryos of different breeder ages, which vary in yolk nutrient availability and for strains that differ in oxygen availability. In the current results, relative liver weight at E18 was lower in high EST than normal EST for embryos of both young and old flocks, but the effect was only statistically significant in the young flock. This might be related to a low yolk nutrient availability. As a result young flock embryos might have low nutrient metabolism and experience a high magnitude of limited energy supply. Therefore the immediate available energy might be prioritized to the demand organ growth in expense of retardation of the liver, which has not reached maximum growth. The same phenomenon also could explain the results at 3 h after hatch, where relative liver weight was lower in high EST than normal EST. These findings are in agreement with Romanoff (1960) who indicated that incubation temperature at 38.5°C resulted in a retardation of liver growth especially during the last d of incubation.

The heart development showed consistent negative response to a high EST, which was found in previous studies (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011; Maatjens et al., 2014) as well as in the current study. The current results show that relative heart weight in embryos at E18 of both young and old flocks was lower in high EST than normal EST. A similar effect also was found for the embryos of both strains, which had a lower relative heart weight in high EST than normal EST. At 3 h after hatch, a 3-way interaction showed that embryos of all groups had a lower relative heart weight in high EST than normal EST and this effect was more pronounced in old Cobb 500 chicks. The plausible explanations of prioritizing limited immediate available energy under high EST for the demand organs might be applicable also for a lower relative heart weight. However, at this moment it is not clear which underlying mechanism is responsible for a higher magnitude in response to high EST of old Cobb 500 chicks.

Development of the gastrointestinal tract, stomach, and intestinal development is inconsistent in response to EST. Stomach and intestinal weights relative to YFBM of embryos at E18 was higher in high EST than normal EST. At 3 h after hatch, there were 2-way interactions between strain and EST for relative intestinal weight and 3-way interactions among breeder age, strain, and EST for relative stomach weight, but these interactions were relatively weak.

Although changes of EST had no effect on hatch of fertile, incubation duration and navel quality revealed that embryos of eggs from different breeder age and strains responded differently to changes of EST. In agreement with previous findings of Nangsuay et al. (2015), at EST of 37.8°C the incubation duration in Cobb 500 was approximately 4 h shorter than Ross 308, especially in the young flocks. This might be explained by a lower eggshell conductance in Cobb 500 than in Ross 308. A low eggshell conductance could lead to a combination of an earlier oxygen-limited stage as previously explained and the difficulty to get rid of CO_2. Consequently at the internal pipping stage the partial pressure of CO_2 (pCO_2) in the air cell is increased (Tona et al., 2010). A high pCO_2 in the air cell at the internal pipping stage is proposed to be a trigger for the hatching process (Visschedijk, 1968). These effects of eggshell conductance were more pronounced at an EST of 38.9°C than at an EST of 37.8°C. The effects of an increased EST to 38.9°C on incubation duration were clearly shown in both young and old flocks of Cobb 500 and Ross 308, but varied in degree of the effect. An EST at 38.9°C in the young flock resulted in a shorter incubation duration of 11 h in Cobb 500 and 9 h in Ross 308, whereas in the old flock incubation duration was shorter by 7 h in Ross 308 and 16 h for Cobb 500. It is not clear why old Cobb 500 chicks appears to be the most sensitive for higher EST in regards to incubation duration. Comparable results were observed for the navel quality. Old Cobb 500 chicks had the highest percentage of chicks with navel score 3, characterized as a black button exceeding 2 mm left outside the navel. A possible reason could be that old Cobb 500 embryos demand more oxygen for nutrient metabolism than young flock embryos due to a large yolk size, whereas the oxygen supply might be less and the CO_2 accumulation in the air cell could be more than old Ross 308. As a result, more yolk nutrient remained unabsorbed, while a high pCO_2 in the air cell triggers the chicks to hatch. A short incubation duration in combination with a high level of

navel score 3 suggests that old Cobb 500 chicks precede the hatching, while the yolk has not been completely absorbed.

Hepatic Glycogen and Blood Metabolites

The measurements of total hepatic glycogen and glucose indicate some responses of embryos to changes of EST, but the pattern of responses was not clear to explicitly explain an influence of strains and breeder ages. Molenaar et al. (2013) demonstrated that a high EST at 38.9°C from E10.5 onward caused an increase in glucose oxidation and decreased hepatic glycogen prior to the hatching process. Nangsuay et al. (2015) reported that at 3 h after hatch Cobb 500 had a higher hepatic glycogen than Ross 308.

The current results showed a 3-way interaction among breeder age, strain, and EST for total hepatic glycogen at E18. The results showed that young Cobb 500 embryos at normal EST had a higher total hepatic glycogen at E18 than old Ross 308 embryos at high EST, whereas the other groups were similar. There was an indication that a high EST at 38.9°C led to numerically low hepatic glycogen at E18, but the same observation did not occur at 3 h after hatch. The current observations regarding high EST suggesting a higher glucose oxidation and lower hepatic glycogen than in a normal EST are in the same direction with Molenaar et al. (2013), but the patterns and mechanisms underlying the responses of embryos from different strains and breeder ages cannot be explicitly explained. An increase in glucose oxidation with high EST might lead to a reduction of substrate for anaerobic glycolysis, which occurs prior to hatching. The current results show that high EST compared to normal EST resulted in a lower plasma lactate in chicks at 3 h after hatch. A similar mechanism of a higher glucose oxidation also could be attributed to a lower plasma lactate in Ross 308 than in Cobb 500. A higher eggshell conductance in Ross 308 than in Cobb 500 can facilitate more oxygen for nutrient metabolism and thereby a reduced glucose availability for anaerobic glycolysis. As a result, plasma lactate of Ross 308 is higher than Cobb 500 at E18 and at 3 h after hatch.

In conclusion, the current results indicate that breeder age has an influence on yolk size, whereas broiler strain has an influence on oxygen availability through eggshell conductance. By incubating eggs of different breeder ages and strains with a normal EST at 37.8°C and high EST at 38.9°C from E7 until hatching, we concluded that oxygen rather than nutrient availability limits embryonic development and growth. Oxygen availability has an influence also in incubation duration and navel quality. The differences in response to EST of embryos from different breeder ages and strains might be related to eggshell conductance. The current results indicate a crucial role of eggshell conductance, which determines embryonic development and growth and influences the magnitude of responses to changes in EST of embryos from different breeder ages and strains. These results suggest that, due to differences in eggshell conductance, embryos from different breeder ages and strains might require different EST to maintain a balance between the availability of oxygen and nutrients, to be able to optimize embryonic development and growth, and good chick quality.

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