Differential physiological response of slow- and fast-growing broiler lines to hypoxic conditions during chorioallantoic membrane development

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ABSTRACT Ambient conditions during chicken embryogenesis, such as insufficient oxygen or changes in temperature, are expected to cause permanent phenotypic changes and affect their posthatch performance. Decades of genetic selection for high growth rate resulted with various physiological and morphological changes that can affect the broiler fitness under environmental stress. To evaluate the selection effect on responses to environmental challenge during embryonic development, and the long-term implications, we have used a unique genetic line, that was not selected for over 30 yr (since 1986), as control for the modern commercial genetic line. At embryonic day 5 (**E5**), broiler embryos from these 2 genetic lines were divided into 2 treatments: 1) control; 2) 15% O_2 concentration for 12 h/day from E5 through E12 the embryonic period of chorioallantoic membrane formation. Embryos and hatched chicks were characterized for physiological and morphological parameters. Significant differences in relative embryo weight and yolk consumption were found between the 2 lines. The modern line was characterized by a higher metabolic rate and rapid growth, supported by higher hemoglobin levels and hematocrit concentrations, whereas the 1986 line had slower metabolism, lower levels of hematocrit and hemoglobin, higher oxygen volume per 1 g of embryonic tissue indicating higher oxygen availability. Both lines exhibited changes in heart rate, and blood parameters corresponding to cardiovascular system adaptation after hypoxic exposure, seemingly implemented to increase oxygen-carrying capacity to the embryo tissues. Our finding stand in agreement that the genetic selection for high growth rate that led to higher metabolism without a fit of the cardiovascular system, increased the imbalance between oxygen supply and demand.

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INTRODUCTION

Since their introduction in the 1950s, genetic selection programs brought dramatic improvements in production traits of commercial broiler stocks (Joseph and Moran, 2005; Zheng et al., 2009; Tavaniello et al., 2014). These selective breeding programs focus on rapid growth, better feed utilization (i.e., lower feed conversion ratio) and higher meat yield (Havenstein et al., 1994, 2003; Zuidhof et al., 2014), and resulted in considerable elevations in feed consumption and metabolism (Sato et al., 2006a,b; Druyan, 2010a). Moreover, Tona et al. (2010) reported different embryonic growth trajectories in various genetic lines. These genetic effects were more pronounced in broiler as compared with layer strains (Tona et al., 2004, 2010). These effects are limited by the tissue biosynthesis rate, which depends, in turn, on the accessibility of nutrients and oxygen consumption during incubation.

Environmental stress during embryogenesis, such as oxygen level and thermal manipulation, is expected to have an extended effect on embryo's growth characteristics and on their posthatch performance (Druyan et al., 2012; Piestun et al., 2008a, 2008b). In light of these observations, Druyan (2010a,b) suggested adjustment and optimization of the incubation environmental conditions to the embryo genetics, to maximize chick hatchability and quality, as well as lifetime performance. Manipulation of environmental conditions during embryo development to improve performance has been evaluated in many studies over the last decade (Piestun et al., 2013a,b; Druyan et al., 2018). Numerous studies had reported that embryos exposed to hypoxia in early

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developmental stages had elevated hematocrit and hemoglobin concentrations that affected their oxygen consumption rate (e.g. Dusseau and Hutchins, 1986, 1988; Ar et al., 1991; Chan and Burggren, 2005; Druyan et al., 2012). Haron et al. (2017) reported that exposure to prolonged and continuous hypoxia, for 72 h during the plateau period (E16-E18), when metabolic processes are increasingly limited by the restricted diffusion capacity of the chorioallantoic membrane (**CAM**), had an adverse impact on embryonic growth and final hatching weight. However, shorter exposures to hypoxia of 12-hour daily enabled the embryo to adequately cope with stress and had no significant influence on embryonic growth and development (Haron et al., 2017).

Ohta et al. (2004) found that embryo growth and protein accumulation, as well as yolk consumption, differed between broilers and layers. Sato et al. (2006a,b) found differences in heat production and lipid metabolism between broiler and layer embryos. The differences in developmental and physiological parameters between broiler and layer embryos are not surprising, considering that broilers have been selected for rapid growth and high meat yield, whereas layers have been selected for egg production. Similarly, Lindgren and Altimiras (2011) demonstrated that embryonic organ growth patterns are differentially affected in broilers and layers when oxygen availability is low. Given that selection for different production traits could manifest in diverged patterns of physiological responses and regulatory mechanisms, it is important to evaluate responses to environmental challenges during embryonic development within each genetic strain. Better understanding of these physiological responses can aid in focusing on more effective ways to improve regulatory plasticity and better support the sustainability of modern broilers.

In this study, we aimed to examine the effects of selection for postnatal growth rate (**GR**) on embryo development, heart rate (**HR**), O_2 consumption, and blood parameters, as well as the effect of exposure to low O_2 concentration (15%) during CAM development (E5-E12) on embryogenesis and metabolism. For this end, we used a comparative analysis, using 2 broiler lines: a modern commercial broiler line and a relaxed broiler line that had not been selected for rapid growth since the mid 1980s.

MATERIALS AND METHODS

The study was approved by the Agricultural Research Organization Committee for Ethics in Using Experimental Animals and has been carried out in compliance with the current laws governing biological research in Israel (approval number: IL-349/11).

Genetic Lines

Two genetic lines were tested in this study: contemporary (2014) commercial Cobb broilers (**Comm**) and a relaxed research line that had been derived from a broiler line used commercially in 1986 (hence called here **L1986**), and maintained since then without any selection for GR or body weight (**BW**).

Experimental Design

All incubation experiments were preformed consecutively in accordance with breeder hen's age. Trial 1 included one incubation experiment (exp. 1) whereas trial 2 included 2 replicated incubation experiments (exp. 2a and 2b). In each of these experiments, 500 fertilized commercial Comm eggs and 500 fertilized L1986 eggs were incubated. In trial 1 (exp. 1), the breeder hens were 50 wk old, with an average egg weight of 66.7 ± 3 g and 58.2 ± 3 g in Comm and L1986, respectively. In exp. 2a, the breeder hens were 54 wk old, with an average egg weight of 69.0 ± 3 g and 61.6 ± 3 g in Comm and L1986, respectively. In exp. 2b, the breeder hens were 60 wk old with an average egg weight of 72.2 ± 3 g and 64.4 ± 3 g in Comm and L1986, respectively.

In all the experiments, eggs were individually numbered and weighed and then incubated in a medium-sized incubator (2,500 eggs) (Danki ApS, Ikast, Denmark) under standard incubation conditions of 37.8°C and 56% relative humidity, with turning once per hour. The incubator was located 31 m above sea level, with 20.9% O_2 in the air. In exp. 2a and 2b on incubation day E5, 250 fertile eggs from each line were randomly assigned to one of 2 treatments: 1) Hypo—O_2 concentration of 15% for 12 h/day from E5 through E12 (250 embryos form each line), or 2) Control—O_2 concentration of 20.9% (250 embryos from each line).

On day E19, all eggs in all experiments were transferred to hatching trays in the control incubator. The experiments were terminated immediately after hatching. Body weight and body temperature (T_b) were measured and blood samples were drawn from the jugular vein of 10 randomly selected chicks from the 4 groups (2 line X 2 O_2 levels) approximately 2 h after hatch (hatching time was recorded) for further analysis.

Low (15%) O_2 Exposure in Trial 2

Exposure to 15% O_2 was accomplished by transferring the eggs to a low O_2 concentration kept in a Medium-Size Incubator (Danki ApS, Ikast, Denmark) equipped with a Model 2BGA-SP-MA O_2 and CO_2 Control System (Emproco Ltd., Ashkelon, Israel); CO_2 level was 0.03 ± 0.01, for 12 h, as previously described by Druyan et al. (2012).

Embryo Measurements

Egg, Yolk, Embryo, and Heart Weights Every day from the offset of the low O_2 challenge (E13) until hatch (E21), 10 random eggs per treatment group were euthanized, and dissected for detailed weight recording using a Type E154 analytical scale (Gibertini, Novate, Italy, ± 0.1 mg). Embryo and hatchling weight are commonly expressed as percentage of the egg weight before incubation, and organ weights are expressed as percentage of

the yolk-free weight of the embryo or hatchling weight, using the following formulas:

$$\text{Relative embryo weight } (\%) = [(\text{embryo weight}) / (\text{initial egg weight})] \times 100$$

$$\text{Relative yolk weight } (\%) = [(\text{yolk weight}) / (\text{initial egg weight})] \times 100$$

liver, breast muscle, and heart of each embryo (or hatchling) were dissected and their weights were used to calculate the relative organ weights, that is, the ratio (%) of organ weight to embryo weight or hatchling weight using the following equation:

$$\text{Relative organ weight } (\%) = [(\text{organ weight}) / (\text{yolk} - \text{free embryo weight})] \times 100$$

Oxygen Consumption O_2 consumption of embryos during incubation was measured daily, from E13 onward, on 5 eggs from each of the 2 lines in exp I and on 4 eggs from each of the treatment groups in exp II. The sampled eggs were placed individually in small cylindrical metabolic chambers, measuring 7 × 7 cm in diameter and height, placed in a water container maintained at 37.8°C. O_2 consumption was measured as previously described (Haron et al., 2017) in an open flow system and calculated using the standard temperature, pressure, dry method in accordance with the following equation:

$$\text{O2 consumption } (\text{mL} / \text{gmin}) = \left[\frac{(20.94(\text{inflow}) - (\text{outflow})) \times 50}{100} \times \frac{60\ min}{embryo\ weight\ g}\right] \times \frac{273^{\circ}K}{(273 + egg\ temperature)^{\circ}K} \times \frac{757}{760}$$

Briefly, dried air was pumped (50 mL/min) into the metabolic chamber, which was fitted with a flow meter scaled from 0-60 mL/min (Aalborg Instruments and Controls, Orangeburg, NY). Dried air from the metabolic chamber was measured for partial O_2 pressure with a model S-3 A/I oxygen analyzer (Ametek, Pittsburgh, PA), and O_2 consumption was measured continuously for 15 min as was previously described by Druyan, 2010a. After the measurement, the embryos in the sampled eggs were euthanized by cervical dislocation and weighed, and the O_2 consumption of each embryo was calculated per 1 g of embryo tissue.

Blood Parameters On each day between E13 and internal pip (**IP**), approximately 0.5 mL of blood was drawn daily from the allantoic vein of 10 embryos per treatment group, into a heparinized syringe. At external pip (**EP**) and after hatch, blood was sampled from the jugular vein. Hematocrit and hemoglobin concentrations in whole blood samples and plasma thyroxin (T4) and triiodothyronine (T3) concentrations in plasma samples were measured. Hematocrit level was estimated by using heparinized microcapillary tubes, the blood in the microcapillary tube was centrifuged in a microliter centrifuge (Hettich, Tuttlingen, Germany) for 8 min at 4,000 × *g*.

Hemoglobin concentration in whole blood samples was determined spectrometrically with a Hemoglobin Reagent Set, Catalog No. H7504 (Pointe Scientific, Canton, MI) in accordance with the manufacturer's instructions. Plasma samples were radioimmunoassayed for total T4 and T3 concentrations, using a Coat-A-Count Canine T4 RIA Kit (Diagnostic Products Corporation, Los Angeles, CA) and an RIA-gnost T_3 Kit (CIS Bio International, France), respectively. The intra-assay and interassay coefficient of variation of the T4 assay were 5.0 and 7.5%, respectively, and those of the T3 assay were 7.8 and 8.2%, respectively.

Heart Rate From E13 onward, HR of 15 embryos from each treatment group was measured daily with a Buddy Digital Egg Monitor (Avitronics, Torquay, UK). Use of infrared transmitters and sensors enabled the amplification of the cardiovascular signal of the embryo within the egg by as much as 20,000 fold, allowing detection of the actual heartbeat of the embryo as early as 12 d after the beginning of incubation. Measurement were taken daily for 90 sec per test under incubation environmental conditions (Druyan, 2010a).

Hatching Time The incubated eggs of exp. 2a and 2b were checked for hatching every 2 h, between hours 460 and 504 of incubation; hatching time for individual chick was recorded. These data were used to calculate hatching duration from the first egg to the last viable hatching chick. The total incubation duration was calculated as the time between setting and emergence.

Hatching percentage was calculated from the number of hatched chicks divided by the number of fertile eggs.

Statistical Analysis

Owing to considerable variation between individual eggs or embryos within lines and treatments across embryonic days, the data from each embryonic day were analyzed separately.

The differences between the 2 lines under standard O_2 (21%) conditions were assessed by combined analysis of all the standard incubation data of exp. 1, exp. 2a, and exp. 2b, using the following two-way ANOVA model:

$$Y = \mu + Line + Incubation\ experiment + Line \times Incubation\ experiment + e$$

with Line (Comm or L1986) and incubation experiment (1, 2a, or 2b) as the main fixed effects, and their interaction.

The effects of hypoxic conditions (low O_2, 15%) vs. standard O_2, and their interaction with the lines, were assessed using trial 2 (exp. 2a and 2b) data and the following 3 factorial (Line, O_2, incubation experiments) ANOVA model:

$$Y = \mu + Line + O_2 + Incubation\ experiment + Line \times O_2 + Line \times Incubation\ experiment + O_2 \times Incubation\ experimente + Line \times O_2 \times Incubation\ experiment + e$$

with Line (Comm or L1986), O_2 (15 or 21%) and incubation experiment (exp. 2a or 2b) as the main fixed effects, and all their interactions.

There were neither significant differences between incubation experiments, nor significant interactions between Line and Incubation experiment (in both ANOVA models), and therefore, the figures show the LSMeans (±SE) of each line calculated from the combined data of all incubation experiments in both trials, whereas the tables show the LSMeans (±SE) of each line-by-O_2 combination in a certain embryonic day, calculated from the combined data of the 2 incubation experiments in trial 2. The Tukey-Kramer HSD test was used for post hoc testing of the differences between the LSMeans of these 4 line X O_2 combinations.

The analysis of hatchability as the proportion of "1" hatched vs. "0" nonhatched individuals within each lines, was tested by chi-square test.

All statistical analyses were conducted with the JMP software (Ver.12) of the SAS Institute (https://www.jmp.com/en_us/home.html).

RESULTS

Effect of Selection For Higher GR and Oxygen Level on Embryo Development: Relative Weights of Body and Yolk

In both lines and both tested O_2 levels, relative embryo weight gradually increased throughout embryogenesis progression. Under standard O_2 conditions, the relative weights of the L1986 embryos were lower at all measured ages (significantly in some ages) compared with Comm embryos, culminating at 61.4 ± 0.47% vs. 57.7 ± 0.46% for Comm and L1986 at hatching, respectively ($P = 0.002$, Figure 1). On days E13 and E14, relative weight of hypoxic L1986 embryos was significantly higher than their L1986 counterparts incubated under standard conditions, whereas no difference

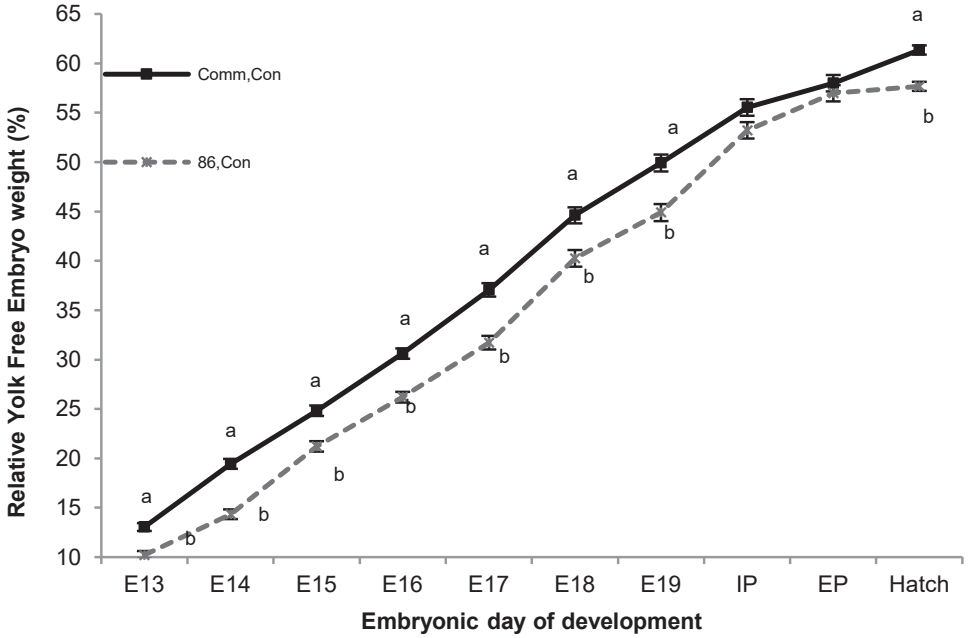

Figure 1. Relative yolk-free embryo weight (percentage of initial egg weight) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

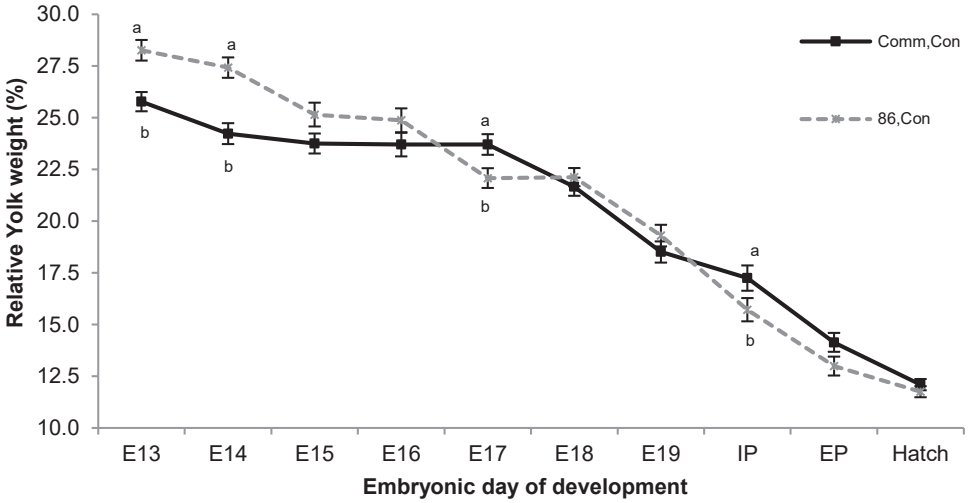

Figure 2. Relative residual yolk weight (percentage of initial egg weight) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

was found between hypoxic and control Comm embryos, and accordingly line-by-O_2 interactions were on the verge of significant (Supplementary Table 1). From day E15 to Ep, hypoxic and control embryos did not differ in relative embryo weight in either line (except E17 in L1986). The hypoxic Comm hatchlings had a significantly lower relative BW in comparison with their control counterparts, whereas no difference was found between hypoxic and standard L1986 chicks; the relative BW of the hypoxic Comm hatchlings was similar to those of the L1986 hatchlings (Supplementary Table 1).

On E13 and E14, mean relative residual yolk weight was significantly higher in L1986 embryos than in Comm embryos, indicating higher yolk consumption by the Comm embryos at these ages (Figure 2). This difference diminished on days E15 and E16 and was reversed on E17 during the "plateau" period and the pipping stages (IP and EP), indicating higher yolk absorption by the L1986 embryos during this time frame. At hatch, mean relative yolk weight of both lines was similar (12.10 ± 0.27% vs. 11.75 ± 0.26% for Comm and L1986 embryos, respectively). Relative yolk weight of hypoxic embryos was similar to their control counterparts.

Effect of Selection for Higher Growth Rate and Oxygen Level on Oxygen Consumption and Blood Parameters

Oxygen consumption gradually increased as embryogenesis progressed. Significantly higher O_2 consumption was observed in Comm eggs on E13 and E14 than in L1986 eggs; it leveled out from day E15 with the progress of the plateau phase in Comm eggs (Figure 3). As the embryo continued to grow and the plateau phase progressed, O_2 demand by the growing embryo increased. However, owing to the peak O_2 diffusion capacity of the CAM at the plateau stage (Druyan et al., 2007), O_2 availability per 1g of tissue declined, as did calculated

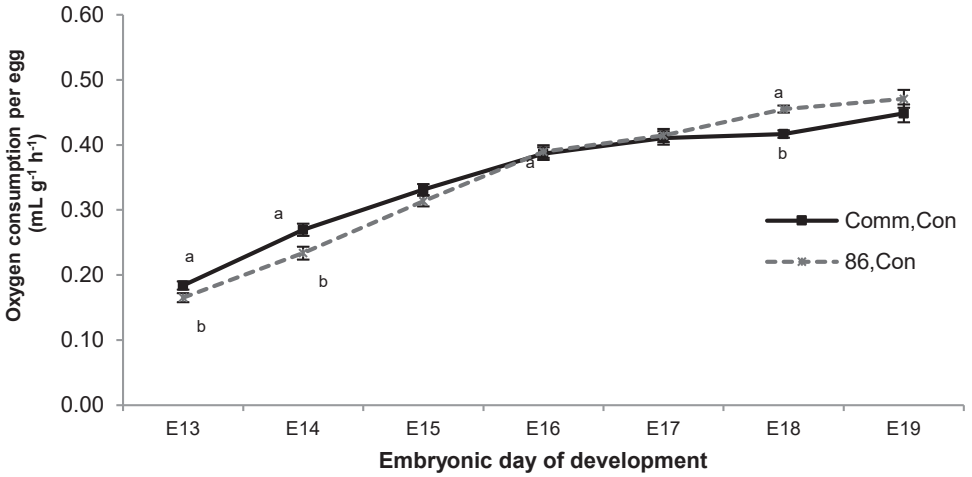

Figure 3. Oxygen consumption (mL g^{-1} h^{-1}) per egg of Comm and L1986 embryos ($n = 13$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

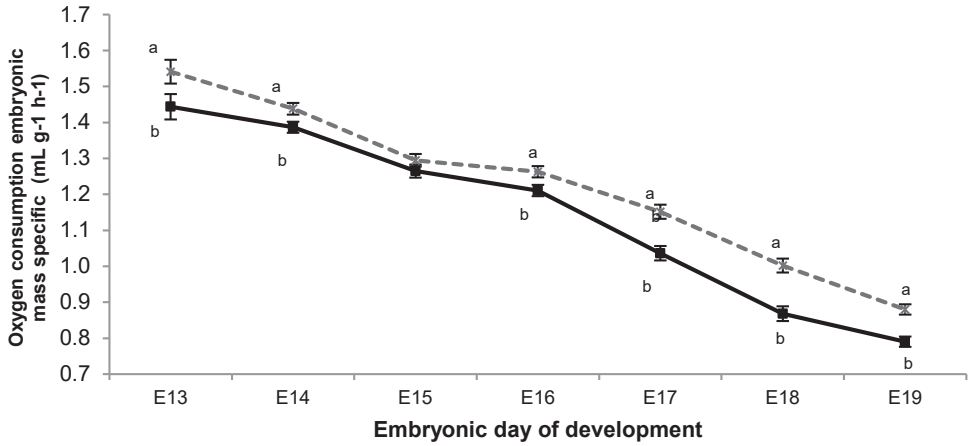

Figure 4. Oxygen consumption (mL g^{-1} h^{-1}) of embryonic mass specific of Comm and L1986 embryos ($n = 13$) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

O_2 consumption per 1g of embryo in both lines (Figure 4). In general, L1986 embryos exhibited higher O_2 consumption per 1g of tissue than Comm embryos from E13 up to E19 (0.88 ± 0.017 vs. 0.79 ± 0.018 mL/gh, on E19, respectively; Figure 4). Oxygen consumption was measured at the end of the 15% hypoxic exposure period, when embryos were maintained under standard incubation condition. No difference in oxygen consumption was found between hypoxic and control embryos within each line (Supplementary Table 2).

In general, Comm embryo hematocrit levels were consistently higher than the hematocrit of the L1986s embryos (Figure 5). In trial 2, hematocrit levels of hypoxic E13 to E19 embryos of both lines, were always higher (significantly in most cases) than their standard O_2 counterparts (Supplementary Table 2). As the embryo continued to grow and approached hatching, hematocrit levels of hypoxic and standard O_2 embryos were similar.

Hemoglobin levels generally correlated with those of hematocrit, with higher hemoglobin concentrations in Comm embryos than in L1986 at most ages. These differences in hemoglobin concentration were found to be significant from E13 up to E17 (Figure 6). However, in both lines, there were neither significant nor consistent differences in hemoglobin concentrations between the standard and hypoxic incubated embryos (Supplementary Table 2).

In both lines, plasma T3 concentrations increased very slightly from E13 through E17, and at each age, were significantly higher in the Comm than in the L1986 embryos (Figure 7). On E18, plasma T3 levels were similar in the 2 lines and on E19, were significantly higher in the L1986 embryos. In both lines, T3 increased dramatically at the IP phase when hatching started, with a significant increase in the hypoxic embryos (∼0.5 ng/mL at E19 vs. 5.5, 5.3 and 5.0 ng/mL at IP, EP and Hatch, respectively, in the L1986 embryos) in comparison with the

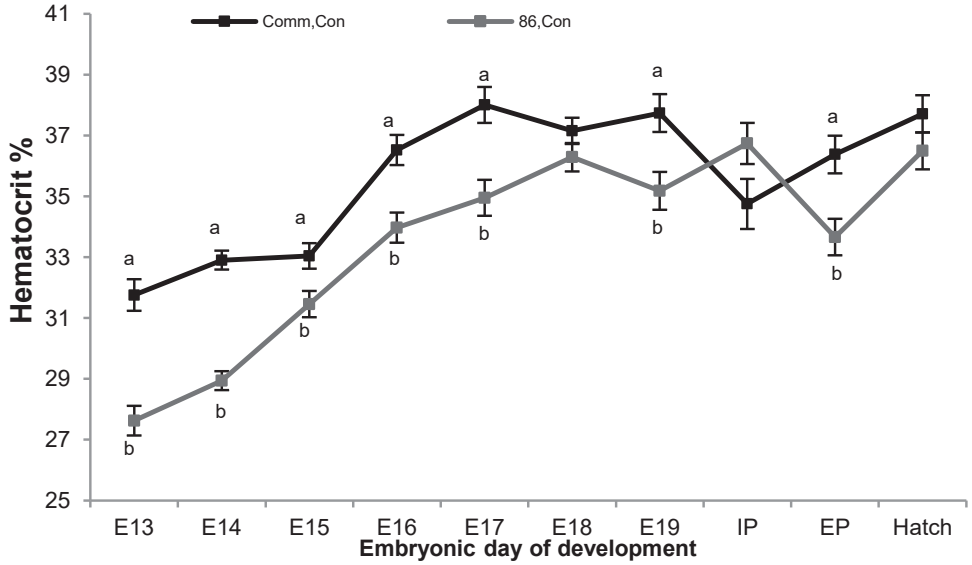

Figure 5. Hematocrit levels (%) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

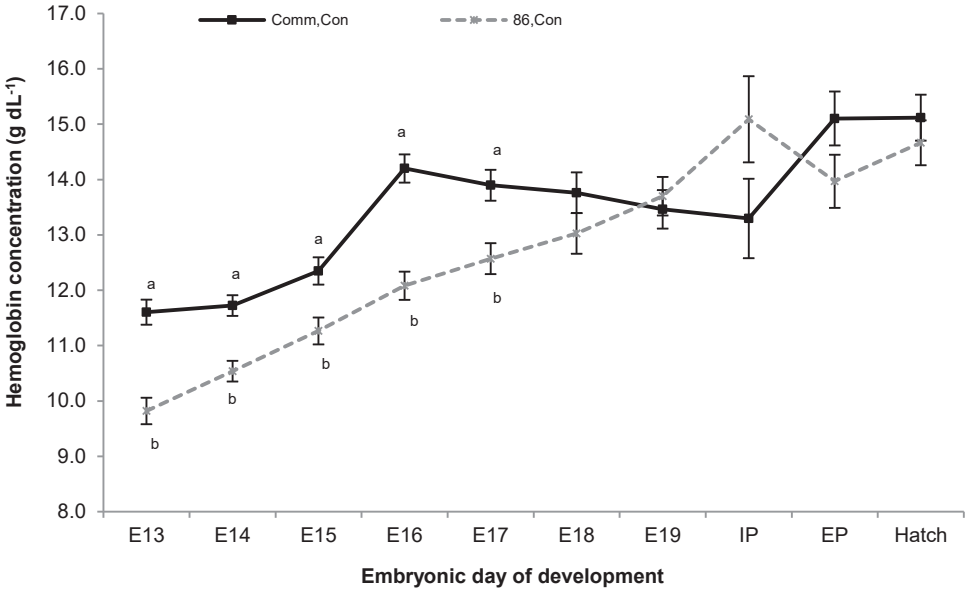

Figure 6. Hemoglobin concentrations (g dL^{-1}) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

Comm embryos (0.3, 3.0, 4.1, and 3.7 ng/mL, at IP, EP and Hatch, respectively; Figure 7). On E13, T3 concentrations in the hypoxic embryos of both lines were significantly lower compare with their standard O_2 counterparts. But from E14 onward, Comm hypoxic embryos exhibited similar to lower T_3 concentrations than their standard O_2 counterparts, whereas L1986 hypoxic embryos had slightly higher T3 levels than their standard O_2 counterpart until E19 (Supplementary Table 3). In L1986 embryos, mean T3 concentrations of hypoxic embryos at IP was significantly lower than standard O_2 embryos, whereas in the Comm embryos, mean T3 concentrations of hypoxic embryos at IP was significantly higher than standard O_2 embryos. In both lines mean T3 concentrations of hypoxic embryos was lower than their control counterparts throughout the EP and hatch stages (Supplementary Table 3).

In both lines, plasma T4 concentrations increased during E13-E16 (Figure 8) and were significantly higher in Comm as compared with L1986 embryos. After reaching the plateau phase, the plasma T4 concentrations were similar in both lines (Figure 8). During IP and EP, T4 concentrations of the L1986 embryos were significantly higher than those measured in the Comm embryos. At hatch, both lines exhibited similar concentrations (4.8 ng/mL vs. 3.8 ng/mL for the Comm and L1986 hatching chicks, respectively). After hypoxic exposure, experimental embryos of both lines exhibited significantly lower T4 concentrations than the control group counterparts on E13. During the last phase of incubation

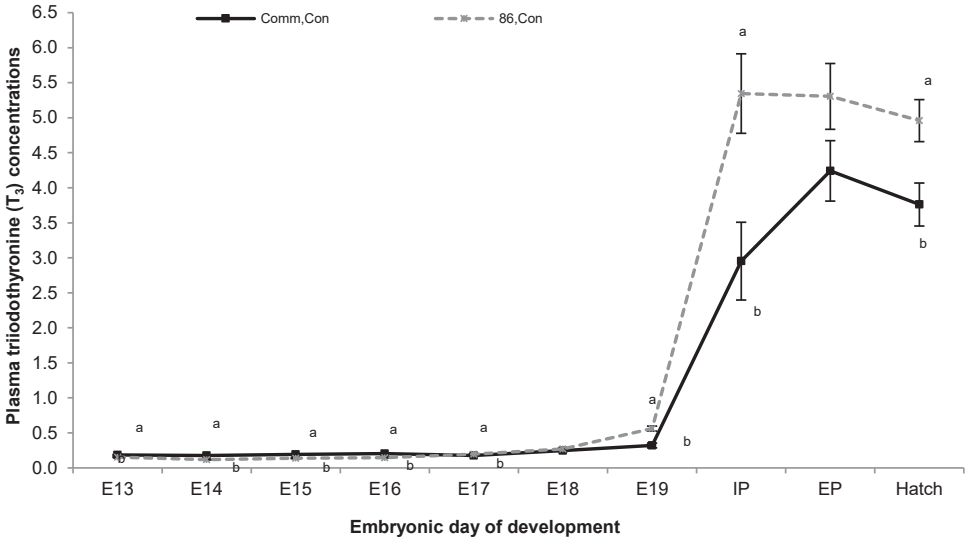

Figure 7. Plasma triiodothyronine (T3) concentrations (ng/mL) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

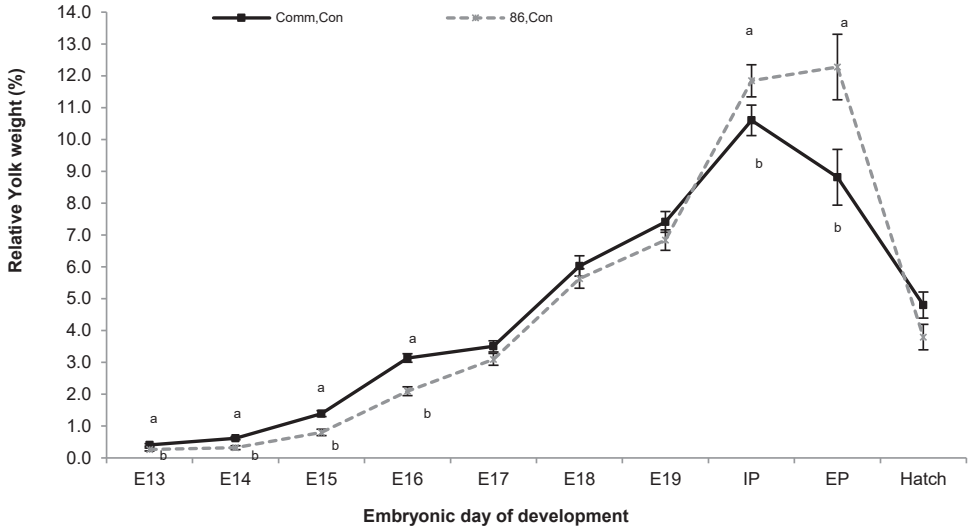

Figure 8. Plasma thyroxin (T4) concentrations (ng/mL) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

T4 concentrations in hypoxic embryos of both lines were lower in comparison with their control counterparts. T4 concentrations of hypoxic Comm embryos was significantly lower from E18 to IP as compared with control Comm embryos, whereas significant T4 level differences between L1986 embryo treatments were only observed during the IP stage. No interline or intertreatment T4 concentration differences were found at hatch (Supplementary Table 3)

Effect of Selection for Higher Growth Rate and Incubation Oxygen Level on Embryo Heart Rate and Heart Weight

From E13 until E19, the HR of Comm broiler embryos was significantly lower than that of L1986 embryos. On E19, HR was similar for both lines (Figure 9). Exposure to hypoxic conditions during the CAM development period led to a reduction in embryo HR in both genetic lines (Supplementary Table 4), which was significantly lower from E13 to E16 in Comm hypoxic embryos as compared with their control counterparts. From E17 to E19, although their HR was lower, no significant difference was found within the Comm line between the 2 incubation treatments. Heart rate of hypoxic L1986 embryos decreased at the end of the hypoxic period and was only significantly lower than their control counterparts on E13 and E14 (Supplementary Table 4). From E15 onward, HR of hypoxia-treated L1986 embryos increased and from E17, it tended to be higher than in control L1986 embryos (Supplementary Table 4).

Relative heart weight ranged between 1.1 and 0.6% as embryogenesis progressed (Figure 10). On most embryonic developmental days (E13, E14, and from E18 up to hatch), Comm broiler embryo relative heart weight was significantly lower than that measured in L1986. Hypoxic exposure affected relative HR and the ratio between heart weight and embryo weight was found to be significantly higher in hypoxic than in control embryos

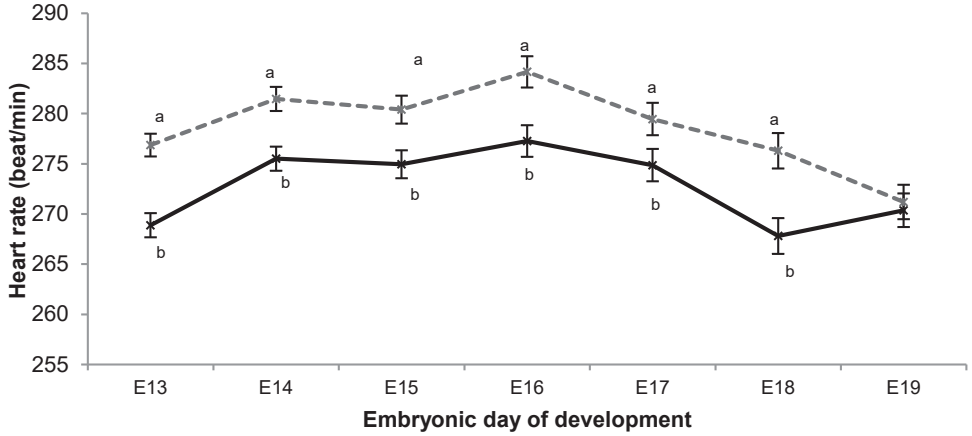

Figure 9. Heart rate (beats/min) of Comm and L1986 embryos ($n = 45$ per line) incubated under standard regime. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

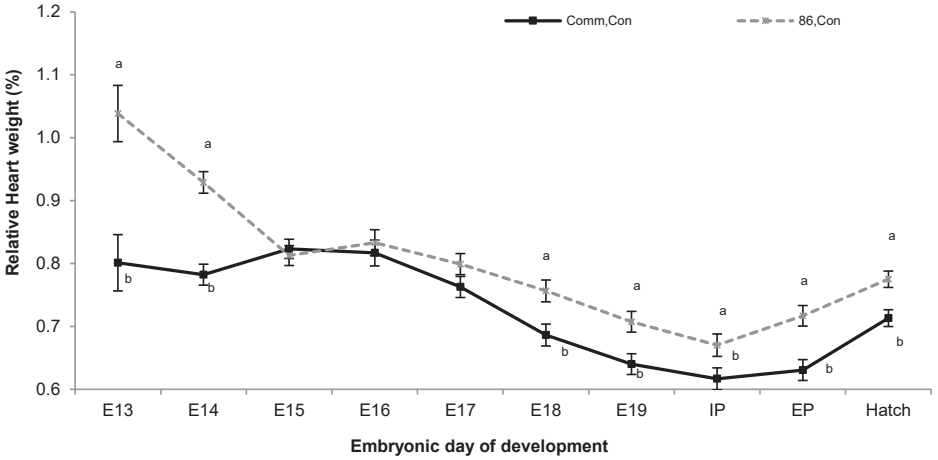

Figure 10. Relative heart weight (% of embryo body weight) of Comm and L1986 embryos ($n = 30$, per line) incubated under standard regimes. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) between treatments.

within both genetics lines on E13 and E15 (Supplementary Table 4), with this difference maintained on E16 and E17 in the L1986 line. On hatch, relative heart weight of hypoxic embryos of both genetic lines did not significantly differ from that of their control counterparts.

Effect of Selection for Higher Growth Rate and Oxygen Level on Hatch

Hatchability was largely similar between lines and incubation treatments: 93.1, 95.3, 96.5, and 94.4% for control Comm, hypoxic Comm, control L1986, and hypoxic L1986 embryos, respectively (Supplementary Table 5). Although first chicks from all Comm treatments groups began to hatch around 476 h from setting, the first L1986 standard-treatment chick hatched at 480 h, whereas the first hypoxia-treated L1986 chick hatched at 482 h from setting. The Comm chicks finished hatching at approximately 506 h, whereas the L1986 chicks finished hatching at around 518 h (Figure 11); mean hatching time of the Comm chicks was 10 h earlier than that of the L1986 chicks. No differences in hatching time were noted between standard and hypoxic incubation conditions of each line.

DISCUSSION

The successful genetic selection of broiler stocks for rapid posthatch growth has also affected their embryonic development. Druyan (2010a,b) reported a significant difference in embryonic development between Cobb, fast-growing Ross broilers and slow-growing Lohmann

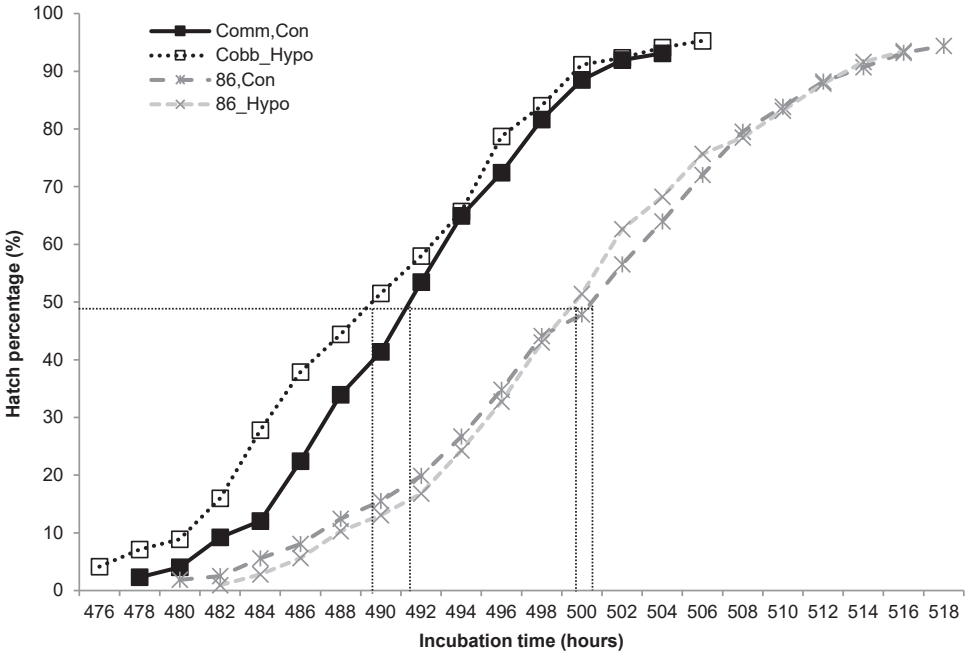

Figure 11. Cumulative hatchability (%) of Comm and L1986 embryos incubated under standard (21% O_2) or hypoxic (15% O_2) regimes.

egg-layers lines, each with distinct overall relative BW, oxygen consumption, and HR.

In chicken embryos, growth and metabolism are limited by oxygen availability, which can be overcome by alteration of cardiac output, redistribution of oxygenated blood (Mulder et al., 1998), increasing blood oxygen-carrying capacity (Dusseau and Hutchins, 1988), modification of hemoglobin (Liu et al., 2009), increasing vascularization (Dusseau and Hutchins, 1989), or any combination of these. Hypoxic manipulation during embryonic development may improve gas diffusion through the eggshell and transport capacity of the blood is expected to be more efficient. Indeed, Druyan et al. (2012) previously reported that exposure to moderate hypoxia of 17% for 12 h daily, from E5 to E12, had a positive impact on CAM development, as manifested by increased ability to deliver oxygen to tissues as compared with the control embryos. Haron et al. (2017) reported an increase in both hematocrit and hemoglobin levels in response to hypoxia of 17% oxygen during the plateau stage (E16-E18), seemingly in attempt to increase oxygen-carrying capacity in an oxygen-deficient environment. In parallel, embryo metabolism decreased, suggesting metabolic adaptation by a decrease in the resting metabolic rate (Haron et al., 2017). This study exploits the difference in embryonic development between modern broiler line and slow-growing relax broiler line (L1986), and challenges their response to moderate hypoxia of 15% O_2 for 12 h daily, from E5 to E12.

While earlier studies have found embryonic weight differences between layers and broilers embryos (Ohta et al., 2004; Everaert et al., 2008; Druyan, 2010a,b; Buzala et al., 2015), this work characterized some of the physiological and endocrinological changes that occurred in fast-growing (Comm) and slow-growing (L1986) broiler lines embryos in response to 15% oxygen hypoxic exposure for 12 h/day during the embryonic period of CAM formation (E5 to E12). The significantly higher relative embryonic weight of Comm vs. L1986 embryos, at most embryonic ages (Figure 1), correlated with the rate and degree of yolk consumption. Plateau stage (E16 to E18) L1986 embryos continued to consume their yolk, whereas the yolk consumption rate of the Comm embryos diminished, yielding similar relative yolk weights at hatch in the 2 lines (Figure 2). Thus, in line with the report of Ho et al. (2011) of a positive relationship between the higher body mass of contemporary broiler embryos and their yolk mass, yolk mass may predict embryo mass during early embryonic development.

The substantial differences in growth and yolk consumption between Comm and L1986 embryos are likely associated with differences in oxygen consumption rates and oxygen demand. In general, oxygen consumption increases with embryonic development and growth during incubation, until E17, after which, it remains relatively constant until E19 (Druyan, 2010a,b). Van Golde et al. (1998) showed that O_2 availability could be a limiting factor for growth as early as the middle of the incubation period, well before metabolic demand exceeds the limitation of the oxygen diffusion capacity of the eggshell. In this study, although Comm embryonic and residual yolk weight data agree with previous literature (Van Golde et al., 1998; Druyan, 2010a,b) and are likely to be limited by O_2 availability, L1986 embryos do not present such dependency in O_2 availability.

During the plateau period, although oxygen consumption remains constant, the metabolic demands of the growing tissues continue to rise until the stage of IP, when the embryos switch to pulmonary respiration. When O_2 consumption was calculated relative to embryo mass, a significant reduction in oxygen availability per gram of embryo mass was found in the Comm as compared to the L1986 embryos (Figure 4). The L1986 embryos, with their lower relative weight, had higher oxygen availability per gram, and lower oxygen supply limitation as compared with Comm embryos, thereby enabling them to continue consuming yolk during this stage.

The lower preplateau relative embryo weight and higher relative residual yolk weight of L1986, compared with Comm, associated with their lower plasma T3 concentrations, that is slower metabolism. During the plateau period, when the Comm broiler embryos had reached an oxygen shortage, their yolk absorption rate diminished and plasma T3 and T4 concentration became similar between the 2 lines. Taken together, Comm embryos with their higher metabolic rate and more rapid growth reach an oxygen shortage already during the embryonic period. This shortage is the result of tissue demand for metabolic energy and oxygen exceeding CAM diffusion and vascular system capacities to adapt and supply oxygenated blood (Tazawa, 1980; Baumann and Meuer, 1992).

The lower metabolism of the L1986 embryos resulted in higher oxygen availability per gram of embryonic tissue, and lower hematocrit and hemoglobin levels as compared with Comm embryos. Druyan (2010a,b) reported on significantly lower hematocrit and hemoglobin levels in embryos from a slow-growing egg-type line compared with commercial broiler embryos. Our results suggest that to compensate for the lack of balance between oxygen availability and demand of the tissues, the vascular system of the Comm embryos response by increasing hemoglobin concentration and hematocrit level.

Heart rate elevation, coupled with elevated stroke volume, is another adaptive response to oxygen shortage (Mortola et al., 2012). Although the available oxygen per gram of embryonic tissue was lower and hematocrit and hemoglobin levels were higher in Comm than in L1986 embryos, Comm embryo HR was lower than L1986, similar to the difference found between broiler vs. layer embryos (Druyan, 2010a,b). This may be due to the blood system adaptation and elevated erythropoiesis implemented to improve oxygen-carrying capacity. Such an increase, if not coupled with plasma volume expansion, may increase blood viscosity, followed by increased blood-flow resistance that can, in turn, lead to a reduction in HR.

While stroke volume was not examined in this study, heart weight is a good indicator of the maximum stroke volume (Ar et al., 1991), that is, a heavier heart demonstrates a higher stroke volume and contraction capacity. In this study, L1986 chicks hatched with significantly heavier hearts compared to Comm chicks. The lower relative heart weight in modern fast-growing broilers and their apparent insufficient oxygen supply, supports the hypothesis that selective pressure for rapid growth and high overall BW have not been accompanied by adjustments in the cardiovascular system that are needed to fully meet the metabolic demands of Comm broilers, even during embryonic development.

Hypoxic conditions during embryonic development can affect embryo metabolism and oxygen supply (Druyan et al., 2012). The actual effects of hypoxia on embryo development depend on the critical period of exposure, hypoxia level, and duration of exposure. While Amaral-Silva et al. (2017) demonstrated that chronic hypoxia (15% O_2) during incubation reduces growth and BW of the hatching chicks, Druyan et al. (2012) found that a daily 12-h exposure to 17% O_2 affected embryo growth, hypoxic embryos had a heavier BW after exposure than that of their control counterparts. In the present study, embryos exposed to 15% O_2 during CAM development had a postexposure BW similar to that of their control counterparts. Only on E13 and E14, hypoxia had a positive influence on L1986 embryo relative weights and they were heavier than their control counterparts; however, at hatching, there were no differences in chicks' weight. Hypoxic exposure seemed to have stronger effect on Comm embryos, as they hatched with a slightly lower relative weight than their control counterparts.

A reduction in GR is considered a survival strategy under hypoxic conditions (Azzam and Mortola, 2007). In the present study, both lines had adapted in order to increase oxygen- carrying capacity to the embryo tissues. After exposure to hypoxic environmental condition, hypoxic embryos from both lines responded by increasing hematocrit level, to better tissues oxygenation (Kohl et al., 2015). However, while for the modern broilers hematocrit levels increased in response to the hypoxic treatments on E13 to E16, the embryos of the 1986 broilers increased hematocrit levels only on E13 and E14. The hemoglobin levels of the Comm embryos had similar pattern of response, whereas no significant pattern in hemoglobin response to hypoxic condition was found in the L1986 embryos.

It appears that the effect of hypoxia on Comm embryos was more severe than on L1986 embryos. In the Comm embryos, the blood system effect persisted through 4 d after exposure, whereas in the L1986 embryos, the effect persisted only for 2 d and was significant only in hematocrit level.

Exposure to hypoxia was found to increase heart mass in domestic chicken and Canadian goose hatchlings (Black and Snyder, 1980; Snyder et al., 1982), which led to ventricular hypertrophy and relatively heavier hearts (Lindgren and Altimiras, 2011). Furthermore, hypoxia caused increased HR and/or stroke volume, which led to increased blood circulation, and thereby imposed changes in oxygen transport (Ar et al., 1991). In the present study, HR and relative heart weights were elevated in response to hypoxic exposure in both lines.

Although Druyan et al. (2012) found no effect of 17% O_2 hypoxia on HR and relative heart weight, hypoxic exposure to a more severe level of 15% oxygen was found to increased relative heart weight and reduce HR of embryos from both lines, the effect persisted until E16 and E18 for the Comm and L1986 lines, respectively. However, although the hypoxic L1986 embryos recovered and their HR returned to normal, the HR of the Comm hypoxic embryos remained lower until E18.

Collectively, the cardiovascular response of Comm embryos to hypoxia may indicate that they are more severely affected than L1986 embryos. It seems that at the initiation of hypoxic stress, Comm embryos were already suffering from an oxygen shortage, which was further exacerbated by the induced hypoxia. Under 15% O_2 hypoxic condition, changes in hematocrit and hemoglobin levels, and the recruitment of new red blood cells were not sufficient to meet the Comm embryo oxygen demands, which ultimately affected the entire cardiovascular system.

The hypoxic embryos from both lines shifted back to standard oxygen conditions (20.94%) at E13. Those chicks had likely adapted to the stress by enhancing their oxygen-carrying capacity, which probably enabled them to supply a higher oxygenated enriched blood to the tissues in comparison with their standard incubated counterparts. Druyan et al. (2012) as well as Molenaar et al. (2010) reported that embryos incubated under low-O_2 conditions converted more residual yolk than their standard incubated counterparts after they were shifted back to normal O_2 conditions. However, in this present study, no compensatory growth or elevated metabolism was observed. The amount of oxygen per gram of embryonic tissue, amount of residual yolk, and plasma T3 and T4 concentrations in the blood of hypoxic embryos within each line were similar to their control counterparts. It seems that 15% hypoxic conditions were too severe for the hypoxic embryos and they needed the extra oxygen to recover and sustain normal growth and development.

The time of external pipping has been shown to advance in response to decreased oxygen content and increased CO_2 content in chicken air sacs (Everaert et al., 2010); consequently a difference in time of hatch exists between broilers and layers (Druyan 2010a,b). In the present study, hypoxic modern (Comm) embryos hatched, on average, 1.5 h before their control counterparts, suggesting that they had not fully recovered and reached their oxygen diffusion capacity limit earlier than the control embryos. In the L1986 line, there was no difference between hypoxic and control embryo hatching times, with most hatching, on average, almost 8 h later than the Comm embryos. It is possible that the delay in hatch time in L1986 embryos was due to their higher oxygen availability, which enabled them also to consume additional yolk

and reach a relative yolk weight similar to that of Comm chicks on hatch.

In conclusion, the genetic selection for high GR that led to higher metabolism without a fit of the cardiovascular system increased the imbalance between the cardiovascular ability to supply oxygen and the growing tissue demand for oxygen. Consequently, the cardiovascular system of the Comm embryos compensate by increasing oxygen-carrying capacity, even under standard incubation condition. Facing hypoxic conditions, the Comm embryos are limited in their ability to properly cope with stress and their embryonic growth and development impaired, compare with the L1986 embryos. After hypoxic manipulation, normoxic incubation condition allows the embryos to continue in their normal developmental trajectory, while their physiological capacity is improved. This can potentially affect the chick posthatch performance and provide the increased oxygen demand of the growing tissues.

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DISCLOSURES

The authors do not have any conflicts of interest to declare.

SUPPLEMENTARY DATA

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