Effects of hypoxic conditions during the plateau period on pre- and posthatch broiler performance

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ABSTRACT Adequate ambient temperature and oxygenation are necessary to maintain normal embryonic development of broilers; however, hypoxia challenge during incubation can aid in improving regulatory plasticity and lead to different phenotypes later in life. This study aimed to examine the effects of moderate hypoxia (O₂ 17%) during the plateau phase on the embryonic physiological parameters and on posthatch performance (growth rate, feed consumption and feed conversion) up to the age of poultry marketing. The study included examined embryos exposed to O₂ 17% for 12 h per day (h/d) from E16 through E18 (designated as 12H), or O₂ 17% continuously, from E16 through E17 (designated as 48H) and a standard incubation control group (21% O₂). Physiological and morphological parameters of embryos and hatched chicks were measured. Male Chicks from all 3 treatment groups were raised under recommended temperature regime, and body weight, feed intake and FCR were recorded on a weekly basis. The intermittent hypoxia protocol (12H), allowed embryos to properly adapt to the shortage of oxygen, compensate for the gap in body mass that developed following the first exposure window, and hatch with characteristics similar to those of the control embryos. In contrast, while the 48H embryos were able to adapt to the hypoxic stress, the prolonged exposure prevented them from catching up with both control and 12H embryos. Broilers that were subjected to hypoxia showed hatching body weights and growth rates similar to those of controls, throughout the entire growth phase. During the fifth wk, lower feed consumption was observed in the 12H and 48H groups and became significantly lower than the control chicks in the sixth wk of growth. Following hypoxia exposure, chicks managed to reach normal body weight with less feed, with the 12H group demonstrating lower and more efficient FCR during the last 2 wk of growth.

Broiler embryos reacted to plateau-phase hypoxia challenge with numerous physiological and metabolic modifications. The prudent alterations in metabolism and cardiovascular system during exposure to hypoxia and posthatch, resulted in more efficient energy utilization in broilers, which may have a long-lasting enhancing effect on posthatching thermotolerance and sustainability in chicks reared under sub-optimal environmental conditions.

Key words: hypoxia, metabolism, plasticity, broiler, incubation

2021 Poultry Science 101:101597
https://doi.org/10.1016/j.psj.2021.101597

INTRODUCTION

The modern broiler has been selected to exploit its full genetic potential to sustain a rapid growth rate and a low feed conversion ratio (FCR). Throughout this evolution, this reduction in FCR led to increased tissue mass at the expense of maintenance (functioning of organs) and thermal regulation (McKechnie, 2008). Moreover, fast growth and increased breast size and weight, is coupled with nonalometric development of major systems, such as the cardiovascular and respiratory systems, that do not undergo the adjustments required to support these fast growth rates (Ben-Gigi et al., 2021). This lack of support impairs broiler ability to maintain adequate dynamic steady state mechanisms, influencing the balance between energy expenditure and demand under suboptimal environmental conditions (Yahav, 2009).

Under optimal environmental conditions, a major part of the energy required for maintenance is expended on the resting metabolic rate (RMR). RMR has been found to exhibit considerable phenotypic flexibility,
associated with physiological adjustment to seasonal acclimation, migration, and short-term thermal exposure (Gelineo, 1964; Hudson and Kimzey, 1966).

Broilers, like all birds, have developed certain responses to cope with environmental stress. The direct responses have been characterized as acclimation (Yahav et al., 1997; Horowitz, 1998), and are based on developmental plasticity during the perinatal period (Yahav and Hurwitz 1996, Nickelmann and Tschenkte 1999, Tschenkte and Basta 2002, Piestun et al. 2008, Druyan et al. 2012a, Haron et al. 2017). Acclimation involves an array of autonomic controlled physiological mechanisms, working in harmony to enhance tolerance to environmental changes (Horowitz, 2002). For instance, acclimation of the cardiovascular system expands the dynamic hypoxic range of the animal, by altering organ response to low ppO2, and inducing a shift in homeostasis to efficiently improve hypoxic tolerance in the new environmental conditions (Horowitz, 2014).

Ambient temperature and adequate oxygenation are necessary to maintain normal development. Altered levels of oxygen, that is, hypoxia or hyperoxia, at diverse time points during embryogenesis, were found to affect fetal development, including body mass and vital organs, depending on the degree, duration, and point of exposure (as reviewed by Haron et al., 2021). In embryos, the primary energy savings during hypoxic exposure are most likely achieved through slowed body growth (Mortola, 2009). While incubation under hypoxic conditions (15% O2) was found to decrease growth rate (GR) and the energy cost of maintenance, incubation under low temperatures was found to only affect GR (Mortola and Cooney, 2008). Further understanding of the response to hypoxia is crucial in order to maximize it positive effect on post hatch performance.

The periods of greatest oxygen demand occur during the last week of incubation, from E15 to E21, when the embryo is utilizing the yolk as its primary energy source (Druyan, 2010). Ultimately, in response to this demand, at around E19-E20, the embryo pips through the chorio-allantoic membrane (CAM) and begins to breathe air inside the shell. Increases in aerobic capacity occur at 2 functional levels that appear to be regulated independently of each other: upregulation of gross energy, and significant elevation in the catalytic activity of the main oxidative control enzymes. Indeed, anaerobic capacity, measured as lactate dehydrogenase activity, is extremely high during early development but diminishes as aerobic capacity increases, beginning at E15 (Seebacher, 2005).

Better understanding these physiological and metabolic responses to hypoxia can aid in focusing on more effective ways to improve regulatory plasticity and lead to different phenotypes later in life (Snyder et al., 1984; Okubo and Mortola, 1988).

This study aimed to examine the effects of exposure of broiler embryos to a moderate O2 concentration (17%) during the plateau phase of embryonic development on the embryonic metabolic rate (MR), cardiovascular parameters, and embryonic development during exposure. In addition, it evaluated the effects of moderate hypoxic exposure (17%) during the plateau phase on the growth rate and feed conversion of broilers maintained under standard growth conditions up to the age of poultry marketing.

**MATERIALS AND METHODS**

The study was approved by the Agricultural Research Organization (ARO) Committee for Ethics in Using Experimental Animals, and was carried out in compliance with the current laws governing biological research in Israel (Approval number: IL-355/11). The experiments were conducted at the ARO poultry farm in Rishon le Zion.

**Embryonic Experimental Design**

The embryonic study included 2 replicated incubation sets. In each of the sets, Fertile Cobb strain broiler chicken eggs (n = 900) with an average weight of 62.0 ± 2.5 g, were obtained from a breeder flock of hens during their optimal period of egg production (35and 38 wk old for the first and second set respectively). In both experiments, eggs were individually numbered, weighed and then incubated in a 2,500 eggs incubator (Danki ApS, Ikast, Denmark) under standard incubation conditions of 37.8°C and 56% relative humidity (RH), with turning once per hour. The incubator was located 31 m above sea level, with 20.9% O2 in the air.

At E16_0 after candling, fertile eggs were randomly assigned to one of 3 treatment groups (300 eggs per treatment):

1. O2 concentration of 17% for 12 h per day (h/d) from E16 through E18 (designated as 12H, those eggs were exposed at 3 time point: E16_0 to E16_12, E17_0 to E17_12, and E18_0 to E18_12).
2. O2 concentration of 17% continuously, from E16 through E17, a total of 48 h (designated as 48H).
3. Control, O2 concentration of 21%.

Exposure to 17% O2 was accomplished by transferring eggs from both hypoxia treatments to an Incubator with 17% O2 equipped with a Model 2BGA-SP-MA O2 and CO2 Control System (Empresco Ltd, Ashkelon, Israel). The O2 sensor activated an electronically controlled pump that infused N2 into the incubator to maintain the desired oxygen concentration of 17 ± 0.2% while CO2 level was 0.03±0.01, as previously described by (Druyan et al., 2012b).

On E19, all eggs from all treatment groups were transferred to hatching trays. The experiments were terminated immediately after hatching.

**Effect of Hypoxia on Posthatch Performance Under Breeder-Recommended Conditions**

**Experimental design** - Fertile Cobb broiler chicken eggs (n = 900) with an average weight of 64.0 ± 2.5 g
were obtained from a breeder flock of hens during their optimal period of egg production (35 wk old). All eggs were numbered and individually weighed prior to incubation. The eggs were incubated and assigned to treatments as described above. One hundred hatching male chicks from each incubation treatment were randomly selected. Each chick was individually tagged and weighed. Chicks were then divided into brooder groups of 10 chicks each, which were raised together in battery cages to the age of 14 d. On d 14, chicks were transferred to individual cages with 2 feeders for every 5 chicks from the same group. Water and feed were available ad libitum, with diet designed to meet the breeder recommendation for broilers. It consisted of a “prestarter” (d 0–10 d), “starter” (11–21 d), grower (21–28 d) and finisher (28d to marketing 43d), with respective contents of crude protein (%) and energy (cal/kg ME) of: 22 and 3,035, 21.5 and 3,100, 20 and 3,180, and 19 and 3,250.

The birds were maintained under the temperature regime recommended by the breeder management guide (starting from 34°C on day of hatch to 23°C from d 21 onward), 55% RH and a 20:4 h light:dark cycle. The chicks were weighed on a weekly basis (n = 100 birds per treatment), and weekly food consumption was calculated for each 10 chicks sharing a feeder (n = 10 biological repeats of feeder group, per treatment). Unadjusted FCR (kg of feed consumed/kg of live BW) was then calculated for each treatment group, per feeder within the treatment.

**Embryo Measurements**

**Heart Rate (HR)** - From E16 to E19, heart rate (HR) of 15 embryos from each treatment group was measured 3 times a day, every 6 h (0 before, 6 and 12 h into the hypoxia exposure), with a Buddy Digital Egg Monitor (Avitronics, Torquay, UK). Use of infrared transmitters and sensors amplify the cardiovascular signal of an embryo within the egg by as much as 20,000-fold, enabling detection of the actual heartbeat of the embryo as early as 12 d after start of incubation (Druyan, 2010).

**Oxygen Consumption** - O₂ consumption by the embryos was measured from E16 to E19, every 12 h before and after the hypoxia exposure. To this end, 5 eggs of each treatment group were placed individually in a small cylindrical metabolic chamber, measuring 7 × 7 cm in diameter and height, which was placed in a water container maintained at 37.8°C. O₂ consumption was measured as previously described (Buffenstein and Yahav, 1991), in an open-flow system. Briefly, dried air was pumped at 50 mL/min into the metabolic chamber, which was fitted with a flow meter scaled from 0 to 60.56 mL/min (Aalborg Instruments and Controls, Orangeburg, NY). Dried air from the metabolic chamber was measured for partial O₂ pressure with a model S-3A/I oxygen analyzer (Ametek, Pittsburgh, PA), and O₂ consumption was continuously measured for 15 min (Piastun et al., 2008; Druyan, 2010). The embryos were euthanized by cervical dislocation at the end of the measurement weight and O₂ consumption was calculated per 1g of embryo tissue, using the standard temperature, pressure, dry (STPD) method according to the following equation:

\[
\text{O}_2 \text{ consumption (mL/g min)} = \frac{(20.94 \text{ (inflow)} - \ ?(\text{outflow})\times50}{100} \times 60 \text{ min embryo weight g}
\]

\[
\times \left(\frac{273}{273 + \text{egg temperature}}\right)^\frac{760}{775}\frac{K}{K}
\]

**Blood Parameters** - Between E16 and E19, 0.5 mL blood was drawn into a heparinized microcapillary syringe, from the allantoic vein of 10 embryos per treatment group, every 12 h, and once on E20 (embryos that had external pip [EP]). Blood for hematocrit measurements was centrifuged in a microliter centrifuge (Hettich, Tuttinglen, Germany) for 8 min at 4,000 × g. Hemoglobin concentration in whole-blood samples was spectrometrically determined using a Hemoglobin Reagent Set (Pointe Scientific, Canton, MI), according to the manufacturer’s instructions. Radioimmunoassays of thyroxin (T4) and triiodothyronine (T3) were performed using commercial radioimmunoassay kits (Diagnostic Products Corporation).

**Egg, embryo, yolk, liver, breast muscle, piping muscle, and heart weights** - Every day, from the initiation of the low O₂ challenge (E16) until hatch (E21), 10 eggs per treatment were euthanized, and then dissected for detailed weight measurement, performed using a Type E154 analytical scale (Gibertini, Novate, Italy, ± 0.1 mg). Since embryo weight is commonly expressed as % of egg weight, the absolute yolk-free weight of each embryo was used to calculate its relative weight (% of initial egg weight), using the following formula:

Relative embryo weight (%)

\[
= \left(\frac{\text{embryo weight}}{\text{initial egg weight}}\right) \times 100.
\]

The yolk (without amniotic fluid and albumen) weight was used to calculate the relative yolk weight with the following formula:

Relative yolk weight (%)

\[
= \left(\frac{\text{yolk weight}}{\text{initial egg weight}}\right) \times 100.
\]

The liver, breast muscle, and heart of each embryo (or chick) were dissected and their weights were used to calculate the relative organ weights, using the following formula:

Relative organ weight (%)

\[
= \left(\frac{\text{organ weight}}{\text{embryo weight}}\right) \times 100.
\]

**Hatching time** - Between incubation h 460 and 504, the eggs were screened every 2 h for hatching, and chick
time of emergence was recorded from each individual egg. The data were used to calculate hatching duration of viable embryos, from the first egg to 100% hatch. The total incubation duration was calculated as the time between setting and emergence. Hatching percentage was calculated. BW and Tb were measured for all hatching chicks.

**Posthatch Measurements**

**Body weight** - Chicks were weighed on a weekly basis using a Sartorius Signum SIWADCP-V14 scale (capacity ± readability 35 kg ± 1 g). Weight gain was calculated as the difference between the current body weight of the chick and the body weight at the previous measurement.

**Feed consumption** - Feed was weighed on a weekly basis, using a Sartorius Signum SIWADCP-V14 scale. Feed consumption per chick was calculated by dividing the weekly feed consumption by the number of chicks at each feeder.

**Feed Conversion Ratio (FCR)** - FCR was calculated by dividing each chick’s feed consumption by its weight gain during the period (each week or the entire growth phase).

**Body Temperature Measurements**: Broiler body temperature was measured at weekly intervals using a digital thermometer (Super Speed Digital Thermometer; Procure Measure Technology Co., San Chung City, Taipei, Taiwan) with ± 0.1°C accuracy that was inserted 1.5 cm into the cloaca. Temperature was measured for 10 chicks per treatment.

**Blood Parameters**: Once a week, approximately 2 mL blood was drawn from the jugular vein of 10 chicks per treatment (1 bird from each feeder group) into a heparinized 23G syringe. Radioimmunoassays of thyroxin (T4) and triiodothyronine (T3) were performed using commercial radioimmunoassay kits (Diagnostic Products Corporation).

**Statistical Design**

**Characterization of the Physiological Response to Hypoxic Manipulation During Incubation** Although HR was recorded as repeated measurements, it was analyzed in a single independent data file with the same generalized linear model that was used to analyze the rest of the data.

Due to considerable variation between individual eggs or embryos within treatments across embryonic days, the data from each embryonic day were analyzed separately. The differences between the treatments (O2 conditions) were assessed by combined analysis of both incubation trials using the following 2-way ANOVA model:

\[ Y = \mu + \text{Treatment} + \text{Incubation} + \text{Treatment} \times \text{Incubation} + e \]

Treatment (Control, 12H and 48H) and Incubation (1 or 2) were the main fixed effects, and their interaction (Treatment × Incubation).

Neither significant differences between the 2 incubation sets, nor significant interaction between Treatment and Incubation set, were found. Therefore, tables and figures show the LSMeans (±SE) of each treatment and on measured embryonic day, calculated from the combined data of the 2 incubation sets. The Tukey-Kramer HSD test was used for post-hoc testing of the differences between treatment LSMeans.

**Effect of Hypoxia on Posthatch Performance**

Individual growth performance data were statistically processed using one-way analysis of variance (ANOVA). \[ Y = \mu + \text{treatment} + e \]

Feed consumption and FCR data were collected for each group of 10 birds (ten replica per treatment), and data were processed statistically using ANOVA

\[ Y = \mu + \text{treatment} + e, \]

With Treatment (Control, 12H and 48H) as the main fixed effect. Values that differed (at a level of \( P \leq 0.05 \)) were considered statistically significant. In addition, a Tukey test was conducted to compare the averages of the treatments.

**RESULTS**

**Effect of Hypoxia on Embryo Growth**

Relative embryo weight gradually increased as embryogenesis progressed (Figure 1). At E17_0, the relative weight of 48H embryos was significantly lower compared to control, while that of 12H embryos was similar to both control and to the 48H treatment groups (33.9 ± 0.8%, 35.6 ± 0.8% and 37.9 ± 0.8% for the 48H, 12H and control embryos, respectively). At E18_0, the relative weight of 12H embryos was significantly lower compared to control, whereas the relative weight of 48H embryos was similar to both its control and the 12H treatment groups. At E18_12, 48H embryos demonstrated lower relative weight compared to both 12H and control embryos. At the EP stage, both hypoxia treatments were associated with significantly lower relative embryo weight as compared to control (58.6 ± 0.8%, 59.3 ± 0.8%, and 62.3 ± 0.8% for the 48H, 12H, and control embryos, respectively). At hatch, the 12H group closed the gap and its relative body weight resembled that of the control group, while the BWs of 48H chicks were significantly lower than control (61.4 ± 0.5%, 63.3 ± 0.5%, and 63.6 ± 0.5% for the 48H, 12H, and control chicks, respectively).

Under hypoxic conditions, reduced embryo growth and subsequently lower BW, was accompanied by deceleration of yolk consumption, as illustrated in Figure 2. Starting from d E17_0 and up to hatching time, with the exclusion of E17_12, 48H embryos had a higher relative yolk weight compared to control embryos. The relative yolk weight of the 12H embryos fluctuated between
being similar to that of the control and to that of the 48H embryos; at E17_0, E18_0, E18_12, and E19_0, the relative yolk weight of 12H embryos was similar to that of both the control and 48H treatment groups. On EP, the 12H relative yolk weight was significantly higher compared to control and similar to that of the 48H group. At hatch, it was significantly lower compared to that of 48H embryos and similar to that of control embryos (13.4 ± 0.4%, 11.6.3 ± 0.4%, and 11 ± 0.4% for the 48H, 12H, and control embryos, respectively).

**Effect of Hypoxia on the Embryonic Cardiovascular System**

HR changes in embryos between E16 and E19 are presented in Table 1. Following hypoxia exposure, the 48H embryos demonstrated a significant elevation in HR as compared to the control. This elevation was noted throughout the entire exposure period, from E16_6 to E17_12. 12H embryos presented variation in HR following hypoxia exposure; on E16 (6 and 12 h into hypoxia), there was a marked elevation in HR, which was found to be significantly higher than in the control, while on E17 (6 and 12 h), the elevation in HR was milder and not significantly different than in control and 48H embryos. At E18_0, 12H embryos had a significantly lower heart rate as compared to controls and was similar to 48H embryos, and at E18_6, the HR was similar to control HR and higher compared to that of the 48H embryos (263.5 ± 3 beat/min, 273.3 ± 3 beat/min, and 271.8 ± 3 beat/min for the E18_6 48H, 12H and control embryos, respectively).
groups, the relative heart weight was not found to be different between the treatments and the control (Table 1).

Both hematocrit and hemoglobin levels (Table 1) were both increased in hypoxia-treated as compared to control embryos. The hematocrit increase was significant in the E18_12 and hatching 12H embryos (39.1 ± 0.6%, 38.3 ± 0.6% and 37.4 ± 0.7% for the 12H, 48H, and control hatching embryos, respectively), as was the hemoglobin increase on E18_0 and E18_12 in both hypoxia-treated groups (13.4 ± 0.5%, 14.7 ± 0.5% and 11.3 ± 0.6% for the 12H, 48H and control E18_0 embryos, respectively), as well as at hatch.

**Effect of Hypoxia on Embryo Metabolism**

O2 consumption per 1 g during the plateau period of embryos in all treatment groups is presented in Table 2. At E18_0, 48H embryos exhibited significantly lower O2 consumption than control and 12H embryos (0.74 ± 0.02 mL/g*h, 0.86 ± 0.02 mL/g*h and 0.84 ± 0.02 mL/g*h for the 48H, 12H and control embryos, respectively). The 12H embryos demonstrated a nonsignificant decrease in oxygen consumption on E16_12 and E17_0 (P < 0.10) and E17_12, which then shifted to a nonsignificant elevation on the last day of exposure.

Changes in T4 and T3 thyroid hormone levels following hypoxia treatments are displayed in Table 2. At E17_0, a significant reduction in plasma T4 concentrations was measured for both hypoxia-treated groups (4.4 ± 0.2 ng/mL, 4.5 ± 0.2 ng/mL, and 5.3 ± 0.2 ng/mL for the 48H, 12H, and control embryos, respectively). At E18_12, 48H embryos had lower T4 concentrations compared to both control and 12H embryos, and on EP, T4 levels in 48H embryos were lower compared to control. At E17_0, and E17_12, 12H plasma T3 concentrations were higher as compared to those measured in control embryos and 48H embryos. At hatch, higher T3 concentrations were measured in both treatments groups as compared to the control, with the difference being significant in the 48H embryos (3.5 ± 0.3 ng/mL, 3.3 ± 0.3 ng/mL, and 2.4 ± 0.3 ng/mL for the 48H, 12H, and control embryos, respectively).

**Effect of Hypoxia on Embryonic Organ Development**

Relative breast weight (Table 3) remained stable throughout the study period, with the exception of E19, when 12H embryos exhibited significantly lower relative breast weight (4.9 ± 0.0%, 4.7 ± 0.06%, and 49 ± 0.06% for the 48H, 12H, and control embryos, respectively). Table 3 displays the relative piping muscle weight, which was significantly higher at E18_0 in the 48H group as compared to the other groups (1.3 ± 0.09%, 1.0 ± 0.0%, and 0.9 ± 0.09% for the 48H, 12H, and control embryos, respectively). No differences were found between the 3 groups with regards to relative embryonic liver weight (Table 3).
Table 2. Oxygen consumption (mL g⁻¹h⁻¹), plasma thyroxin (T4) and plasma triiodothyronine (T3) concentrations (ng/mL) of control (Con), 12H and 48H embryos incubated under different oxygen regimes from E16 through E18.

| Parameters          | E16_0     | E16_12    | E17_0     | E17_12    | E18_0     | E18_12    | E19_0     | EP    | Hatch |
|---------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-------|-------|
| Oxygen consumption  | 0.94 ± 0.02 | 0.91 ± 0.02 | 0.95 ± 0.02 | 0.84 ± 0.02 | 0.84 ± 0.02 | 0.79 ± 0.02 | 0.81 ± 0.02 |       |       |
| (mL/g/h)            | 0.02      | 0.02      | 0.02      | 0.02      | 0.02      | 0.02      | 0.02      |       |       |
| Plasma thyroxin (T4) | Con 3.93 ± 0.14 | 4.91 ± 0.42 | 5.32 ± 0.19a | 5.94 ± 0.36 | 7.22 ± 0.51 | 9.72 ± 0.57a | 11.17 ± 1.24 | 14.02 ± 0.65a | 3.26 ± 0.66 |
| (ng/mL)             | 12H**      | 4.55 ± 0.41 | 4.59 ± 0.19b | 6.08 ± 0.38 | 7.08 ± 0.51 | 9.43 ± 0.54a | 8.41 ± 1.24 | 12.33 ± 0.65ab | 3.91 ± 0.62 |
|                     | 48H***     | 4.42 ± 0.20b | 5.71 ± 0.36 | 7.06 ± 0.51 | 7.91 ± 0.57b | 8.93 ± 1.30 | 11.61 ± 0.87b | 3.79 ± 0.66 |       |
| &p; (f)             | ns         | 0.0272     | ns         | ns         | 0.0421     | ns         | 0.056     | ns    |       |
| Plasma triiodothyronine (T3) | Con 0.269 ± 0.01 | 0.268 ± 0.02 | 0.285 ± 0.02ab | 0.324 ± 0.2ab | 0.319 ± 0.02 | 0.398 ± 0.03 | 0.567 ± 0.08 | 5.46 ± 0.73ab | 2.479 ± 0.27ab |
| (ng/mL)             | 12H**      | 0.229 ± 0.2 | 0.371 ± 0.03ab | 0.335 ± 0.02b | 0.360 ± 0.02 | 0.393 ± 0.03 | 0.451 ± 0.08 | 4.081 ± 0.73b | 3.84 ± 0.27b |
|                     | 48H***     | 0.350 ± 0.02ab | 0.283 ± 0.02b | 0.307 ± 0.02 | 0.390 ± 0.04 | 0.391 ± 0.09 | 3.655 ± 0.73b | 3.53 ± 0.27b |       |
| &p; (f)             | ns         | 0.0444     | 0.0459     | 0.0906     | ns         | 0.0245     | 0.024     |       |       |

* Different letters indicate significant differences (P ≤ 0.05) between treatment groups on a given day of incubation.
* Hypoxic exposure started on time EX_0 and ended 12 h later at EX_12, on the same exposure day for 12H treatment.
*** Hypoxic exposure started on E16_0 and ended on E18_0 for 48H treatment.

Table 3. Relative breast weight, piping muscle weight and liver weight of control (Con), 12H and 48H embryos incubated under different oxygen regimes from E16 through E18.

| Parameters          | E16_0     | E16_12    | E17_0     | E17_12    | E18_0     | E18_12    | E19_0     | EP    | Hatch |
|---------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-------|-------|
| Relative breast     | Con 6.02 ± 0.09 | 5.80 ± 0.08 | 5.23 ± 0.08 | 5.33 ± 0.10 | 4.94 ± 0.13 | 4.85 ± 0.11 | 4.99 ± 0.07a | 4.68 ± 0.11 | 4.71 ± 0.11 |
| weight (%)          | 12H**      | 5.95 ± 0.09 | 5.50 ± 0.08 | 5.36 ± 0.11 | 5.10 ± 0.13 | 4.96 ± 0.11 | 4.71 ± 0.07b | 4.75 ± 0.11 | 4.94 ± 0.10 |
|                      | 48H***     | 5.32 ± 0.08 | 5.29 ± 0.10 | 5.11 ± 0.13 | 5.08 ± 0.11 | 4.94 ± 0.07b | 4.77 ± 0.11 | 4.60 ± 0.10 |       |
| Relative piping     | Con 0.49 ± 0.06 | 0.82 ± 0.08 | 0.95 ± 0.09b | 1.23 ± 0.10 | 1.59 ± 0.19 | 1.57 ± 0.13 | 1.54 ± 0.17 |       |       |
| muscle weight (%)   | 12H**      | 0.60 ± 0.06 | 1.02 ± 0.09 | 1.01 ± 0.09b | 1.28 ± 0.10 | 1.68 ± 0.19 | 1.70 ± 0.13 | 1.47 ± 0.16 |       |
|                      | 48H***     | 0.78 ± 0.06 | 0.91 ± 0.08 | 1.31 ± 0.09b | 1.23 ± 0.10 | 1.68 ± 0.19 | 1.85 ± 0.13 | 1.61 ± 0.16 |       |
| Relative liver      | Con 2.43 ± 0.10 | 2.34 ± 0.07 | 2.46 ± 0.08 | 2.38 ± 0.10 | 2.33 ± 0.05 | 2.32 ± 0.06 | 2.13 ± 0.08 | 2.30 ± 0.08 | 2.40 ± 0.09 |
| weight (%)          | 12H**      | 2.26 ± 0.08 | 2.52 ± 0.08 | 2.22 ± 0.10 | 2.41 ± 0.05 | 2.25 ± 0.06 | 2.33 ± 0.08 | 2.35 ± 0.08 | 2.40 ± 0.08 |
|                      | 48H***     | 2.57 ± 0.08 | 2.44 ± 0.10 | 2.29 ± 0.05 | 2.28 ± 0.06 | 2.18 ± 0.08 | 2.29 ± 0.08 | 2.49 ± 0.08 |       |

* Different letters indicate significant differences (P ≤ 0.05) between treatment groups on a given day of incubation.
* Hypoxic exposure started on time EX_0 and ended 12 h later at EX_12, on the same exposure day for 12H treatment.
*** Hypoxic exposure started on E16_0 and ended on E18_0 for 48H treatment.
Effect of Hypoxia on Hatching Parameters

In the 12H treatment group, 50% of the hatchings occurred 2 h earlier than in the 48H and control groups (Figure 3). All chicks hatched with a similar body weight (46.15, 46.46, and 46.29 for Con, 12H and 48H respectively), but when removing residual yolk, the body weight of hypoxia-exposed chicks was significantly lower than that of control chicks. The hatching chicks of both treatment groups also had a lower Tb as compared to control (39.7 ± 0.02°C, 39.7 ± 0.02°C and 39.8 ± 0.02°C for the 48H, 12H, and control embryos, respectively) (Table 4).

Posthatch Performance

The mean weekly chick body weight in all groups was similar throughout the entire growing period (Figure 4). Likewise, the mean weekly posthatch weight gain (Figure 5) and the mean weekly FCR (Figure 7) were similar in all groups. Significant differences were found in mean weekly feed consumption during the last week of growth, with chicks of both hypoxic treatment groups consuming less food than the control chicks (9,590 ± 391 g, 9,233 ± 391 g and 11,073 ± 391 g for the 12H, 48H and control chicks, respectively; Figure 6).

Control, 12H, and 48H chicks had similar Tb throughout the growing period (Table 5).

Changes in T4 and T3 thyroid hormone levels during the growing period are displayed in Table 5. Hypoxic incubation conditions were found to have no effect on T3 concentrations on 7 to 14 d old broiler. On d 21, a significant difference in plasma T3 concentrations was found between broilers from both hypoxic groups and controls (2.09 ng/mL, 1.67 ng/mL, and 1.66 ng/mL for control, 12H, and 48H chicks, respectively; Table 5). A similar pattern was found on d 28 and d 35. On d 42, broilers from all incubation groups had similar level of T3. T4 concentrations were similar between all 3 groups throughout the growing period. Final BW was similar across groups as was the FCR (Table 6), but was slightly lower in both treatment groups (1.77, 1.73 and 1.75 for control, 12H, and 48H chicks, respectively; p(f) = 0.10).

DISCUSSION

Hypoxia during broiler embryo incubation has been found to trigger adaptation of the embryonic cardiovascular system to the altered environment, with elevations in blood parameters, such as hematocrit, hemoglobin (Haron et al. 2017) and HR (Tomi et al., 2019). The actual effects of hypoxia on embryo development depend on the critical period of exposure, hypoxia level, and duration of hypoxic exposure. This work examined the physiological changes that occurred in broiler embryos subjected to daily 12-h hypoxia (17% O2) for 3 consecutive days (from E16 through E18) as compared to continuous hypoxia exposure for 48 h, from E16 to E18. These embryonic days are considered the period of plateau in oxygen availability for the entire egg and are
characterized by an increase in oxygen demand and limited diffusion capacity of the CAM (Druyan et al., 2007).

The effect of hypoxia exposure during the plateau period of embryonic development on the cardiovascular system was significant and rapid, primarily affecting embryo HR; 6 h into hypoxia, a significant increase in HR was observed compared to controls. Elevation in HR in response to lower oxygen availability has already been documented by Tomi et al. (2019). While the 48H group demonstrated a higher HR than control embryos throughout the entire hypoxia period, the 12H embryos shifted from HR higher to similar to controls, which directly correlated with the hypoxia timing. When the embryos were returned to standard conditions (21% O₂), HR declined. Moreover, as exposure progressed, 12H embryo responses got milder, which may have been due to improved adaptation of the circulatory system to the recurring hypoxic stress or to a decrease in embryonic demands for oxygen. The significant changes in HR of the hypoxic embryos were not accompanied by variations in relative heart weight, as previously described by Rouwet et al. 2002.

Hypoxia during embryonic development was reported to affect growth and metabolism of embryos, contingent on the hypoxia regimen. Amaral-Silva et al. (2017) showed that 15% O₂ during the last third of incubation led to lower hatchling body mass. Similarly, Dzialowski et al. (2002) reported that eggs exposed to 14.4% O₂ during either the second or last third of incubation, had significantly smaller embryo masses. However, embryos exposed to hypoxia during the first third...
of incubation recovered and closed the gap in body mass. In the current work, the 48H treatment was associated with lower BW at hatch, while embryos subjected to 12H hypoxia treatment had the same BW at hatch as control, which lay in agreement with observations presented by Haron et al. 2017. Body mass of hypoxia-reared Salmon’s alevins was comprised of higher yolk mass, but with a lower yolk-free body mass compared to normoxia-reared alevins (Polymeropoulos et al., 2017), as was seen in this study following both hypoxia treatments, particularly the 48H regime. The normal BW of the 12H group at hatch following the delay of development during incubation was an expression of the ability of the chicken embryo to maintain normal growth, despite hypoxic stress. The intermediate hypoxia protocol (12H), allowed them to properly adapt to the shortage of oxygen, compensate for the gap that developed after the first exposure window, and arrive to hatch with characteristics similar to those of the control embryos. In contrast, while the 48H embryos adapted to the hypoxia stress, the prolonged exposure prevented them with catching up with the control and 12H embryos, as shown by their inferior hatching phase profiles.

In general, oxygen consumption in embryos increases with embryonic development and growth up until E17 and then remains approximately constant in the plateau phase, until E19 (Druyan, 2010). Following either 12H or 48H hypoxia exposure, embryos exhibited a decrease in oxygen consumption compared to controls. The

Figure 6. Mean weekly feed consumption (g) in control (Con), 12H and 48H chicks (n = 10 feeding group per treatment) incubated under different oxygen regimes between E16 and E18, from day of hatch through marketing age of 42 d.

Figure 7. Mean weekly feed conversion ratios (FCR) in control (Con), 12H and 48H chicks (n = 10 feeding group per treatment) incubated under different oxygen regimes between E16 and E18, from day of hatch through marketing age of 42 d.
identified modifications in thyroid hormone levels were further indications of metabolic adaptation following hypoxia exposure during the plateau period. The thermoregulatory response is mainly mediated by the level of metabolism induced or supported by the thyroid hormone axis. On E17, both T3 and T4 levels were significantly reduced in 48H embryos, and although T3 is the potent hormone with regards to embryonic metabolism, the fact that plasma T4 concentration was also significantly reduced suggests that there was an overall reduction in thyroid gland activity. This finding is in agreement with Piestun et al. (2009), who observed a decrease in thyroid gland activity following embryo exposure to thermal manipulation (39.5°C) during incubation.

Various studies have shown that hypoxia during embryogenesis affects fetal development, including a substantial effect on vital organs. Embryos exposed to 15% oxygen hypoxia for a period of 6 d (E1–E6, E6–E12, or E12–E18) were developmentally retarded and smaller compared to controls (Chan and Burggren, 2005). In this study, neither heart nor liver weights were significantly affected by exposure to low oxygen levels. In contrast, Itani et al. (2016) reported that although embryos remained with weights similar to controls following exposure to more severe hypoxia, heart weight was lower. The maintained liver weight was in agreement with Määtä et al. (2018), who found no deviation in liver weight in mice subjected to hypoxia. Breast muscle weight was similar between the treatments and the control throughout the measuring period, except for E19_0, when it was lower in the 12H group, but then equalized at the next measurement, indicating that the embryos compensated for slower growth towards hatch. At E18_0, the 48H embryos had significantly higher relative piping muscle weight compared to both 12H and control embryos, indicating early preparation for hatch as a result of the hypoxic stress. This finding contrasts the decline measured in response to thermal manipulation during incubation (Piestun et al., 2009). Overall, despite slight differences in organ weights, such as the breast muscle and piping muscle, upon exposure to hypoxia, embryos generally compensated for the changes before hatch.

Hypoxia challenge during the plateau phase did not have a negative effect on the hatching chicks; hatch percentage and chick BW were similar across all 3 groups. The 12H group reached 50% hatching 2 h earlier than the other 2 groups, which may have been directly triggered by the recurrent hypoxia, as previously reported by Druyan et al. (2012b).

Table 5. Plasma thyroxin (T4) and plasma triiodothyronine (T3) concentrations of control (Con), 12H and 48H male chicks (n = 10 per treatment, one chick from each feeder group) incubated under different oxygen regimes between E16 and E18, from hatch through 42 d.

| Parameters     | 7 d       | 14 d      | 21 d      | 28 d      | 35 d      | 42 d      |
|----------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Temperature (°C) | Control 40.9 ± 0.08 | 41.0 ± 0.05 | 41.4 ± 0.05 | 41.4 ± 0.05 | 41.5 ± 0.10 | 41.5 ± 0.09 |
|                | 12H 40.8 ± 0.08 | 41.0 ± 0.05 | 41.4 ± 0.05 | 41.3 ± 0.06 | 41.4 ± 0.10 | 41.4 ± 0.09 |
|                | 48H 40.8 ± 0.08 | 40.9 ± 0.05 | 41.4 ± 0.05 | 41.4 ± 0.06 | 41.3 ± 0.10 | 41.4 ± 0.09 |
| p (f)          | ns        | ns        | ns        | ns        | ns        | ns        |
| Plasma thyroxin (T4) concentrations (ng/mL) | Control 7.84 ± 0.72 | 9.00 ± 0.51 | 5.00 ± 0.55 | 7.68 ± 0.052 | 10.93 ± 0.59 | 11.70 ± 0.77 |
|                | 12H 7.59 ± 0.75 | 9.09 ± 0.51 | 7.05 ± 0.55 | 7.29 ± 0.52 | 10.70 ± 0.59 | 9.79 ± 0.77 |
|                | 48H 6.51 ± 0.76 | 9.46 ± 0.48 | 6.99 ± 0.58 | 8.07 ± 0.52 | 10.49 ± 0.62 | 10.08 ± 0.77 |
| p (f)          | ns        | ns        | ns        | ns        | ns        | ns        |
| Plasma triiodothyronine (T3) concentrations (ng/mL) | Control 1.43 ± 0.13 | 1.71 ± 0.10 | 2.90 ± 0.13a | 1.69 ± 0.10 | 1.35 ± 0.11a | 0.95 ± 0.08 |
|                | 12H 1.37 ± 0.13 | 1.72 ± 0.10 | 1.67 ± 0.13a | 1.47 ± 0.10 | 1.30 ± 0.11a | 0.94 ± 0.08 |
|                | 48H 1.48 ± 0.13 | 1.63 ± 0.10 | 1.66 ± 0.13a | 1.41 ± 0.11 | 0.97 ± 0.11b | 1.07 ± 0.08 |
| p (f)          | ns        | ns        | ns        | 0.0465    | 0.0459    | ns        |

Table 6. Final body weights and feed conversion rates (FCR) of male broiler, from control, 12H and 48H, treatments, at day of marketing (43 d).

| Incubation treatment | Mean final body weight (g) | FCR |
|----------------------|----------------------------|-----|
| Control              | 3137.9 ± 27.93             | 1.77 ± 0.03 |
| 12H                  | 3141.7 ± 27.93             | 1.73 ± 0.03 |
| 48H                  | 3096.2 ± 28.35             | 1.75 ± 0.03 |
| p (f)                | ns                         | =0.10 |

Means ± SD are presented.

Posthatch Performance of Broilers Subjected to Hypoxia During Embryonic Development

Since the 1950s, commercial genetic selection programs have brought to a dramatic improvement in broiler production traits, including rapid growth, high feed utilization and higher meat production characteristics (Havenstein et al. 1994, 2003; Zuidhof et al. 2014). Genetic selection for performance traits also resulted in considerably increased MR, which has, in turn, altered growth mechanisms and development (Druyan, 2010). Such developments logically necessitate parallel increases in the size of the cardiovascular and respiratory systems, as well as enhancements in their functional efficiency. However, insufficient development of these major systems has led to the relatively poor ability to adequately balance energy expenditure and body water
homeostasis under extreme environmental conditions (Yahav, 2009). Indeed, modern broilers have an elevated MR and consequently elevated internal heat production, resulting in insufficient maintenance of thermoregulation processes, and ultimately to larger body temperature fluctuations.

$T_h$, which is considered to be an effective indicator of metabolic heat production and later heat tolerance (De Basilio et al., 2003), was significantly lower at hatching in 12H and 48H broilers as compared to control. On d 21, 28 and 35, 12H and 48H chickens exhibited decreased T3 and similar T4 plasma concentrations than controls. Thyroid hormones are known to regulate heat production in mammals and in avian species (Loyau et al., 2013). The combination of lower $T_h$ and lower plasma thyroid hormone concentrations in the 12H and 48H broilers suggested that metabolic plasticity (Haron et al., 2021) and/or decreased heat production (Piestun et al., 2008; Collin et al., 2011) may underlie the responses to hypoxic exposure.

Nonetheless, broilers that were subjected to plateau-phase hypoxia showed normal hatching BW and growth rates similar to those of controls, throughout the entire growth phase. An alternative beneficial response might consist of a combination of reduced maintenance energy demand coupled with an overall reduction in energy consumption. During the first 4 wk of growth, chicks in the treatment and control groups consumed the same amount of feed; however, in the fifth wk, lower feed consumption was observed in the 12H and 48H groups and was significantly lower than the control chicks in the sixth wk of growth, when the broiler demands for energy peak. The lower feed consumption in the 2 treatment groups suggest lower energy demands of these broilers, namely, they managed to reach similar body weight with less feed. Even though the FCR was similar between all groups, the 12H group demonstrated lower and more efficient FCR during the last 2 wk of growth ($p(f) < .10$).

To conclude, broiler embryos reacted to plateau-phase hypoxia challenge with numerous physiological and metabolic modifications. The exposure to hypoxia did not have a negative effect on the hatching chicks and advantage was observed in posthatch performance at the late stage of growth, when treated broilers consumed less food without damaging their growth, and even demonstrated a trend of enhanced FCR. The alterations in metabolism and cardiovascular system during exposure to hypoxia and posthatch, resulted in more efficient energy utilization in broilers, which may have a long-lasting effect on enhanced posthatching thermotolerance and sustainability when reared under sub-optimal environmental condition. The mechanisms underlying such long-lasting effects remain to be further elucidated, especially in relation to metabolic plasticity.

DISCLOSURES

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

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