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Hematology from embryo to adult in the bobwhite quail (*Colinus virginianus*): Differential effects in the adult of clutch, sex and hypoxic incubation

Josele Flores-Santin[a,*], Maria Rojas Antich[b], Hiroshi Tazawa[b], Warren W. Burggren[b]

[a] Laboratorio de Eco-Fisiología, Facultad de Ciencias, Universidad Autónoma del Estado de México, Instituto Literario # 100. Col. Centro, Estado de México C.P. 50000, Mexico

[b] Developmental Integrative Biology, Department of Biological Sciences, University of North Texas, 1155 Union Circle #302220, Denton, TX 76203, USA

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ABSTRACT

Hematology and its regulation in developing birds have been primarily investigated in response to relatively short-term environmental challenges in the embryo. Yet, whether any changes induced in the embryo persist into adulthood as a hematological form of "fetal programming" is unknown. We hypothesized that: 1) chronic as opposed to acute hypoxic incubation will alter hematological respiratory variables in embryos of bobwhite quail (*Colinus virginianus*), and 2) alterations first appearing in the embryo will persist into hatchlings through into adulthood. To test these hypotheses, we first developed an embryo-to-adult profile of normal hematological development by measuring hematocrit (Hct), red blood cell concentration ([RBC]), hemoglobin concentration ([Hb]), mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, as well plasma osmolality. Hct, [RBC] and [Hb] in normoxic-incubated birds (controls) steadily increased from ~22%, ~1.6×10^6 μL^{-1} and ~7 g% in day 12 embryos to almost double the values at maturity in adult birds. Both cohort and sex affected hematology of normoxic-incubated birds. A second population, incubated from day 0 (d0) in 15% O_2, surprisingly revealed little or no significant difference from controls in hematology in embryos. In hatchlings and adults, hypoxic incubation caused no significant modification to any variables. Compared to major hematological effects caused by hypoxic incubation in chickens, the hematology of the bobwhite quail embryo appears to be minimally affected by hypoxic incubation, with very few effects induced during hypoxic incubation actually persisting into adulthood.

1. Introduction

Fetal experiences during prenatal development in vertebrates can affect subsequent juvenile and adult phenotype and consequently cause potential morphological, physiological and other effects after birth and later in life. This phenomenon, termed 'fetal programming', is being extensively studied in human and domestic mammals (for recent reviews see Alexander et al., 2015; Roberts et al., 2015; Sedaghat et al., 2015; Stangenberg et al., 2015; Nilsson and Ling, 2017; Itani et al., 2017). This potentially pathological phenomenon is typically associated with some form of insufficiency in placental-derived nutrition. In contrast to the intimate relationship between the growing fetus and the maternal environment in mammals and other viviparous animals, in avian eggs, the development of embryos occur without any direct physiological or behavioral maternal influences. Consequently, avian embryos can directly experience variations in the environment, which offers a tractable experimental model for studying "embryonic programming". For instance, development of avian embryos during the very earliest blastocyst stages soon after egg fertilization can be experimentally manipulated by changes in environmental temperature and duration of pre-incubation egg storage (Funk and Biellier, 1944; Lundy, 1969; Butler, 1991; Wilson, 1991; Branum et al., 2016; Itani et al., 2017; Reyna and Burggren, 2017). Consequently, these experiences prior to the onset of incubation reflect subsequent phenotype of prenatal embryos ("embryonic programming'). Indeed, altered thermal environment and/or pre-incubation egg storage period in the very early blastocyst stages of development of chicken embryos diminish blood oxygen carrying capacity and interfere with acid-base balance when measured at day 15 (d15) of development (Haque et al., 1996; Branum et al., 2016). Later in development, hypoxic incubation, at least in the

* Corresponding author at: Laboratorio de Ecofisiologia, Facultad de Ciencias, Universidad Autonoma del Estado de Mexico, Instituto Literario # 100. C.P. 50000 Col. Centro, Toluca, Mexico.
E-mail address: jfloress538@profesor.uaemex.mx (J. Flores-Santin).

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chicken and the quail, result in changes both morphological and physiological traits (Flores-Santin and Burggren, 2015).

Of course, not all avian species have similar incubation histories or conditions. Some embryos are constantly incubated from time of laying (e.g. chickens), while some species lay their eggs over a period of time before gathering them together to start incubation (e.G. *zebra* finch, bobwhite quail, ostrich) (Gilby et al., 2013; Reyna and Burggren, 2012; Wilson, 1991). Some fossorial bird embryos likely even experience natural hypoxia during incubation (e.g., bank swallow, burrowing owls) (Boggs et al., 1983, 1984; Burggren et al., 1994; Tazawa et al., 1994) or temporary water inundation of the nest (e.g., grebes) (Sotherland et al., 1984).

Most environmental effects on avian embryonic traits have been determined in chicken embryos, which has long been a model for development (Burggren et al., 2015). This is especially true for the investigation of hematology (for recent review see Bílková et al., 2017). Consequently, we simply do not know if the responses of this domesticated bird are typical of all birds, or perhaps more typical of birds who as embryos experience little if any environmental fluctuation. The Bobwhite quail (*Colinus virginianus*) lives in environments that are frequently characterized by highly variable conditions, including exposure of the eggs to both high temperatures and low humidity (Reyna and Burggren, 2012). Exposure to hyperthermic temperatures during early stages of pre-incubation results in aberrant development, variability in incubation length and reduced hatch rates in bobwhite quail (Reyna and Burggren, 2012; Reyna and Burggren, 2017). As such, this species might be expected to show relatively plastic hematology and other developmental processes compared to, for example, the domestic chicken in response to environmental variables.

An additional factor that might affect hematology of the quail is sex. A meta-analysis of 36 studies providing mean and variance estimates for hematocrit (Hct) for each sex showed no difference between male and female birds (Fair et al., 2007). However, some studies show that, while in some species Hct was significantly higher in male than female, in other species female Hct was higher. Even in the same species, there is conflicting evidence regarding the relationship between sex and Hct (Carey and Morton, 1976; Morton, 1994). Another source of variation that is not often considered is differences between cohorts (flocks) of birds. Therefore, in the current study we analyze the effects of sex on hematological variables we obtained for different cohorts in bobwhite quail.

We hypothesized that hematological variables such as hematocrit (Hct), red blood cell concentration ([RBC]) and hemoglobin concentration ([Hb]) will be increased in quail embryos incubated in or exposed to hypoxia. We additionally hypothesized that these early hematological effects will persist into the adult quail as a form of "embryo programming", reflecting limited developmental physiological plasticity with respect to restoration of the normal phenotype.

To test these hypotheses, we first quantified hematological variables, i.e. Hct, [RBC], [Hb] and mean corpuscular indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration ([MCHb])) in developing bobwhite quail embryos, hatchlings, juvenile and adult birds. We also assessed osmolality (Osm) as an indicator of MCV regulation. It was assumed that Hct, [RBC] and [Hb] increase with age during embryonic development and after hatching through juveniles to adults, as occurs in other related birds (Grima and Girard, 1981; Mueller et al., 2015). Such baseline data for the bobwhite quail have not been previously reported in a single study across all of development. After establishing this baseline, we investigated the effects of hypoxic incubation and its potential embryonic programming on hematological variables in hatchlings. We compared quail incubated as embryos in air (20.5% O_2, 50% RH) with those incubated in chronic hypoxia (15% O_2) and subsequently measured as hatchlings, juvenile or adults. Additionally, we investigated the effect of hypoxic exposure on hematological regulation in d16 quail embryos by determining responses of hematological variables to 24 h exposure to 15% O_2 and comparing them with published values in d15 chicken embryos at similar developmental stage. Finally, sex-based differences in hematology of developing birds have not been extensively studied. If such differences exist, they could be a complicating factor adding to the existing variability in the data. Hence, we determined sex-based differences in hematological variables of adult quail.

2. Materials and methods

2.1. Protocols

All experiments were conducted at the University of North Texas in accordance with the protocol 1212–20 approved by the UNT Institutional Animal Care and Use Committee. Fertile eggs of bobwhite quail (*Colinus virginianus*) in six cohorts were obtained from two different local commercial hatcheries - Strickland Game Birds, Pooler, GA, USA and Lake Cumberland Game Bird Farm, Monticello, KY, USA. Each named cohort came from a single hatchery, but within a cohort eggs came from different female birds at that hatchery. Cohorts were received in the months from July, September and November. The intent was to use multiple cohorts to generate a complete fertilization-to-senescence hematology profile and determine how hypoxic incubation altered this profile. The hatchery of origin of each egg was recorded, and unexpected but interesting differences between the hematology of cohorts and hatcheries subsequently emerged, and became part of the study.

Eggs were weighed (± 0.01 g) and incubated in air at a temperature of 37.5 ± 0.1 °C and a relative humidity (RH) of ~50% (incubator model 1502, G.Q.F. Manuf. Co., USA). On d11 of incubation, eggs were candled to discard non-embryonated eggs. Then, viable individual eggs were removed from the incubator on each of d12 through d20 (i.e., prenatal embryos) and d21 (internally pipped or IP embryos) for blood collection from the allantoic vein or artery located by candling on the previous days. The IP stage was identified visually after blood collection on d21. Blood was then collected to measure hematological variables, as described below. Insufficient blood could be collected for hematological analysis due to collapse of the chorioallantoic membrane blood vessels in externally pipped (EP) embryos, so eggs were allowed to hatch and the blood was collected from hatchlings on the following day, 1 day post-hatch (referred to as 1 dph).

2.1.1. Normoxic populations ("Norm1" to "Norm3")

Prenatal embryos developing from d12 to d20, IP embryos and 1 dph hatchlings – all developing in normoxia (air) - were designated as the '*Norm1*' group (Table 1). They were regarded as a single cohort comprising multiple clutches (i.e. multiple breeding pairs) from the same flock (Strickland Game Birds, Pooler, GA, USA). N values at each day of development for this group are shown in Fig. 1.

Two additional cohorts of quail eggs were incubated and hatched in air (designated as *Norm2 and Norm3*, Table 1). Hatchlings were reared in an incubator (Hatchrite Corp., Kirkwood, MO) thermostatted at 37 °C and ~50% RH for 31 days and then transferred to rearing cages, where they received quail diet and water ad libitum. The rearing room had a 12 h light and dark cycles at ~4 lx and a temperature of ~22 °C and RH of ~35–40%. The *Norm2* group was sampled for blood analysis on the following days (with N values in parentheses): 15 dph (8), 30 dph (9), 110 dph (7) and 222 dph (7). The other cohort, *Norm3*, which was designed to extend towards senescence, was sampled for blood analysis at ages 100 dph (10), 193 dph (15), 283 dph (11) and 424 dph (11).

2.1.2. Hypoxic incubation effects in prenatal embryos, juveniles and adults ("ChronicHypo")

An additional cohort of birds (designated as *ChronicHypo*) comprised eggs incubated in chronic hypoxia (15% O_2/85% N_2) together with a companion group incubated in normoxia (air) to serve as control

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