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RESEARCH ARTICLE

Manipulating plasma thyroid hormone levels at hatching alters development of endothermy and ventilation in Pekin duck (*Anas platyrhynchos domestica*)

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ABSTRACT

At hatching in precocial birds, there are rapid physiological and metabolic phenotypic changes associated with attaining endothermy. During the transition to *ex ovo* life, thyroid hormone levels naturally increase, peaking at hatching, and then decline. To better understand the role of the natural increase in thyroid hormone at hatching in regulating the developmental trajectory of the Pekin duck's endothermic phenotype, we examined development of O_2 consumption ($\dot{V}_{O_2}$) and ventilation (frequency, tidal volume and minute ventilation) while inhibiting the developmental increase in thyroid hormones that occurs at hatching via administration of the thyroid-peroxidase inhibitor methimazole (MMI) or accelerating the developmental increase via triiodothyronine (T_3) supplementation. Animals were dosed only on day 24 of a 28-day incubation period and studied on incubation day 25, during external pipping (EP) and 1 day post-hatching (dph). On day 25, there was an increase in $\dot{V}_{O_2}$ in the hyperthyroid treatment compared with the other two treatments. During the EP stage, there was a significant effect of thyroid status on $\dot{V}_{O_2}$, with hyperthyroid $\dot{V}_{O_2}$ being highest and hypothyroid $\dot{V}_{O_2}$ the lowest. By 1 dph, the supplemented T_3 and control animals had similar $\dot{V}_{O_2}$ responses to cooling with comparable thermal neutral zones followed by increased $\dot{V}_{O_2}$. Hypothyroid 1 dph hatchlings had a lower resting $\dot{V}_{O_2}$ that did not increase to the same extent as the supplemented T_3 and control animals during cooling. During EP, inhibiting the rise in T_3 resulted in embryos with lower ventilation frequency and tidal volume than control and supplemented T_3 embryos. At 1 dph, ventilation frequency of all animals increased during cooling, but tidal volume only increased in supplemented T_3 and control hatchlings. Our data support the role of the late incubation increase in T_3 in regulating the systemic development of endothermic metabolic capacity and associated control of ventilation occurring at hatching of the Pekin duck.

KEY WORDS: Development of endothermy, Metabolism, Triiodothyronine, Ventilation development

INTRODUCTION

Thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are key regulators of vertebrate development and metabolic physiology, mediating coordinated developmental changes in various tissues. Thyroid hormones play prominent roles in vertebrate metabolism and seasonality; for example, salmon migration, cold acclimation in fish and anuran metamorphosis and development are affected by thyroid hormone levels (Holzer and Laudet, 2015). Thyroid hormones have been argued to be fundamental regulators required for the evolution of endothermy (Little et al., 2013; Little and Seebacher, 2014). In birds and mammals, thyroid hormones are key metabolic regulating hormones that affect growth, maturation, aerobic metabolism, body temperature and mitochondrial function (Chaffee and Roberts, 1971; Collin et al., 2005; Decuypere et al., 2005; McNabb, 1992).

Thyroid hormones exert their effects on all tissues and increase the demand for oxygen by increasing energy expenditure (Mullur et al., 2014). In juvenile birds, lack of thyroid hormones are associated with decreased growth, while increased thyroid hormone levels are associated with elevated metabolic rates (McNabb, 1992; McNabb and Darras, 2014). Thyroid hormones play a critical role in regulation of mammalian and avian thermogenesis to maintain high and constant body temperature (T_b) and adaptive thermogenesis during exposure to cold ambient temperature (T_a) (Danforth and Burger, 1984; McNabb et al., 1998).

Endothermy develops rapidly in precocial hatchlings like the Pekin duck (Sirsat et al., 2016; Sirsat and Dzialowski, 2016). Previous studies show that ducklings significantly increase resting oxygen consumption ($\dot{V}_{O_2}$), heat production, mitochondrial respiration and heart mass immediately after hatching (Sirsat et al., 2016). During the peri-hatch period in precocial birds, the levels of circulating plasma thyroid hormones peak (McNabb, 2007). There is a correlation between the rise in thyroid hormone levels at hatching and physiological changes associated with increased metabolic capacity and endothermy. This correlation points towards a role for thyroid hormones in mediating maturation of endothermy, the respiratory system and skeletal muscle function associated with increased aerobic metabolism (De Groef et al., 2013).

The aim of the current study was to investigate how altering the timing of the increase in plasma T_3 levels would influence hatching and the development of endothermy and ventilatory control during this critical developmental window of hatching in Pekin ducks. We pharmacologically manipulated total plasma T_3 levels during the late embryonic stage of development just prior to the animal attaining an endothermic phenotype, hatching and beginning locomotion. We measured aerobic metabolism, body temperature and pulmonary ventilation in both the thermoneutral zone and during gradual cooling. We found a strong relationship between thyroid hormone status and alterations in metabolism, ontogeny of endothermy, pulmonary ventilation and time to hatching. With these experiments, we tested the hypothesis that the rise in thyroid hormone occurring at hatching is required for proper development of the endothermic and ventilatory responses to cooling. Observations in this study support

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List of abbreviations and symbols

dph	days post-hatching
D25	day 25 embryo
EP	external pipping
f	breathing frequency
f_H	resting heart rate
$F_{E_{O_2}}$	excurrent O_2 fraction of dry gas
$F_{I_{O_2}}$	incurrent O_2 fraction of dry gas
MMI	methimazole
Pa_{mean}	intrinsic mean arterial pressure
T_3	3,3′,5-triiodo-L-thyronine
T_4	thyroxine
T_a	air temperature
T_b	body temperature
T_s	body-surface temperature
TNZ	thermal neutral zone
$\dot{V}_{O_2}$	oxygen consumption
$\dot{V}_E$	minute ventilation
V_T	tidal volume

the important role that the increases in thyroid hormones occurring during the perinatal period play in regulating ontogeny of endothermy, hatching and development.

MATERIALS AND METHODS

Egg incubation and animal care

Eggs of Pekin ducks (*Anas platyrhynchos domestica* Linnaeus 1758) were purchased from Blanco Industries (McKinney, TX, USA), weighed and incubated at 37.5°C and 60% relative humidity in an egg incubator (model 1202; GQF Manufacturing, Savannah, GA, USA). Eggs were turned automatically every 4 h. At the internally pipped stage, eggs were relocated to a clear Lyon incubator maintained at 37.5°C and 70% relative humidity to allow for hatching. Time-lapse images of the eggs were taken with an Olympus camera (SP-590UZ) every 30 min during hatching to determine time of hatch, which was then used to determine age of each hatchling. Eggs were candled daily from incubation day 25 (D25) onwards to determine advancement of development (external pipping and mortality). Hatchlings were kept in a Hatchrite incubator at 32°C and provided with water and food *ad libitum*. At embryonic day 24 (D24, ∼86% of incubation) of a 28-day incubation period, eggs were randomly assigned to 3,3′,5-triiodo-L-thyronine (T_3; hyperthyroid), methimazole (MMI; hypothyroid) or 0.9% saline (control) treated groups. Measurements were made on Pekin duck embryos on D25, during external pipping (EP) and 1 day post-hatch (dph). All procedures were approved by University of North Texas Institutional Animal Care and Use Committee.

Drug administration

On D24 of incubation to manipulate T_3 levels prior to hatching, developing embryos were treated with either T_3 (Sigma-Aldrich, St Louis, MO, USA), MMI (a thyroid-peroxidase inhibitor in thyroid gland; MP Biomedicals, Solon, OH, USA) or 0.9% NaCl solution (control). Solutions were injected onto the inner membrane at the air cell of eggs on D24 of incubation around 12.00 h, as in Sirsat et al. (2018). At this stage of embryonic development, the thyroid gland is differentiated and the hypothalamic–pituitary–thyroid axis is functional in duck and chicken (McNabb, 2007). Before injection, egg viability was confirmed by candling, the air cell was marked with a pencil, and eggs were moved to a benchtop incubator (1602N HovaBator, GQF Manufacturing) maintained at 37.5°C. The eggshell was cleaned with 70% isopropyl alcohol wipes and a hole (∼1.3 mm) was made in the shell above the air cell using a sterile 18G needle. All injections were made into the air cell using a 100 µl pipette (Gilson Inc., Middleton, WI, USA). T_3 (200 µg kg^{-1}, D24 egg mass dose) was prepared by dissolving it in sterile 0.02 mol l^{-1} NaOH and diluting with 0.9% NaCl sterile saline. MMI (10 mg kg^{-1}, D24 egg mass dose) was prepared by dissolving it in 0.9% NaCl sterile saline. Identical volumes of 0.9% NaCl sterile saline solution were injected onto the inner membrane of the air cell in control eggs. Drug injections were given in a microliter volume equal to the mass in grams of the egg (i.e. a 55 g egg was injected with 55 µl of the drug solution). After injection, the shell was cleaned with 70% isopropyl alcohol and the hole was sealed with silicone gel (DAP Products, Baltimore, MD, USA). The eggs were then returned to the egg incubator. On subsequent days, embryo viability, pipping stages and mortality were recorded. Egg mass before incubation did not differ among T_3, MMI and control eggs (Table 1).

Whole animal metabolic rate

Oxygen consumption ($\dot{V}_{O_2}$), a proxy of metabolic rate, was measured using flow-through respirometry during gradual cooling as in Sirsat and Dzialowski (2016). Animals were placed in respirometry chambers (∼500 and 1000 ml) that were housed inside a programmable insulated incubation cabinet set to 37.5°C for embryos and 35°C for hatchlings. Animals were acclimated for at least 60 min prior to the beginning of the cooling protocol. To test for the metabolic response to cooling, the incubation cabinet temperature was gradually decreased at a rate of 9.2°C h^{-1} until ambient temperature (T_a) reached 20°C for embryos and 15°C for hatchlings. A gaseous mixture between 20.9 and 21.3% oxygen balanced with nitrogen was mixed by a microprocessor control unit (controller model 0154, Brooks Instruments, Hatfield, PA, USA) and flow controllers (model 5850E, Brooks Instruments). This mixture flowed into each respirometry chamber at a known flow rate measured with a calibrated FlowBar1 mass flow meter (Sable Systems International, Las Vegas, NV, USA). A portion of outflow gas from the respirometry chamber was pulled by a pump (R1 flow controller, AEI Technologies, Pittsburgh, PA, USA) through a Nafion tube (AD Instruments, Colorado Springs, CO, USA) covered in Drierite, a column of Sodasorb and another Nafion tube for water and CO_2 removal before it was pulled through an O_2 analyser (FC-10, Sable Systems). Sampling of outflow gas from multiple respirometry chambers was automatically controlled by a custom-built solenoid multiplexer controlled by LabChart 7 software (ADInstruments, Colorado Springs, CO). A maximum of three animals was measured at any given time and each chamber was sequentially sampled for 150 s. An extra solenoid was used for sampling of inflow O_2 level. Data were recorded with a PowerLab 16SP and LabChart 7 software. Rates of oxygen consumption ($\dot{V}_{O_2}$, ml O_2 min^{-1}) were calculated using the following equation (Withers, 2001):

$$\dot{V}_{O_2} = \dot{V}_I \times \frac{(F_{I_{O_2}} - F_{E_{O_2}})}{1 - F_{E_{O_2}}}, \quad (1)$$

where $\dot{V}_I$ is incurrent flow rate (ml min^{-1}), $F_{I_{O_2}}$ is the incurrent O_2 fraction of dry gas, and $F_{E_{O_2}}$ is the excurrent O_2 fraction of dry gas.

Animal body temperature was continuously recorded during respirometry measurements. In embryos inside the egg, body-surface temperature (T_s) was measured just under the eggshell. Eggs were candled and marked with a pencil to determine the embryo and

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Table 1. Morphological measurements at day 25, external pipping (EP) and 1 day post-hatch of control, T_3-supplemented and MMI-treated Pekin duck (*Anas platyrhynchos domestica*)

	Age	Control	T_3	MMI
Initial egg mass (g)	D25	91.2±7.7 (12)	91.8±5.4 (10)	92.7±9.0 (16)
	EP	91.1±4.7 (17)	91.5±6.1 (9)	87.5±5.3 (10)
	1 dph	95.3±9.3 (10)	95.2±3.7 (10)	87.5±12.5 (5)
Yolk-free body mass (g)	D25	37.72±7.28^{a} (13)	41.45±6.31^{a} (9)	40.28±11.08^{a} (16)
	EP	51.35±2.71^{b} (17)	52.23±3.13^{b} (11)	50.43±4.37^{b} (10)
	1 dph	55.75±4.94^{b} (11)	53.33±4.26^{b} (10)	49.82±6.12^{b} (5)
Yolk sac mass (g)	D25	18.27±2.07^{a} (12)	19.48±2.44^{a} (9)	18.94±4.23^{a} (15)
	EP	11.76±2.28^{b} (17)	10.88±1.81^{b} (11)	9.18±2.90^{b} (10)
	1 dph	4.35±1.41^{c} (11)	6.34±2.33^{c} (10)	5.18±2.07^{c} (5)
Cardiac ventricle mass (g)	D25	0.143±0.03^{a,b} (13)	0.167±0.03^{a} (9)	0.143±0.04^{b} (16)
	EP	0.222±0.03^{c,d} (16)	0.252±0.03^{c} (11)	0.233±0.04^{d} (8)
	1 dph	0.383±0.05^{e,f} (11)	0.381±0.05^{e} (10)	0.340±0.08^{f} (5)
Liver mass (g)	D25	0.657±0.13^{a} (13)	0.692±0.12^{a} (9)	0.688±0.17^{a} (16)
	EP	0.977±0.08^{b,c} (17)	1.08±0.09^{b} (11)	1.44±0.36^{c} (9)
	1 dph	1.80±0.19^{d} (11)	1.7±0.23^{d} (10)	1.95±0.30^{d} (5)
Right femur length (mm)	D25	17.25±1.69^{a} (13)	17.37±1.58^{a} (9)	17.1±2.28^{a} (16)
	EP	20.61±0.8^{b} (17)	20.35±0.55^{b} (9)	20.57±1.41^{b} (9)
	1 dph	22.33±0.9^{c} (10)	21.70±0.54^{c} (10)	21.13±1.17^{c} (5)
Head to beak length (mm)	D25	38.72±1.41^{a} (13)	38.40±1.19^{a} (9)	38.05±1.83^{a} (16)
	EP	41.50±0.91^{b} (17)	42.25±0.86^{b} (11)	41.25±1.5^{b} (9)
	1 dph	44.98±1.96^{c} (10)	43.29±1.0^{c} (10)	42.82±1.06^{c} (5)
Hematocrit (%)	D25	43±4^{a} (10)	42±3^{a} (7)	41±3^{c} (12)
	EP	38±3^{b} (17)	40±4^{b} (9)	35±5^{d} (8)
	1 dph	40±5^{b} (11)	39±3^{b} (10)	36±3^{d} (5)

Sample sizes are provided in parentheses. Similar superscript lower case letters (or no letters) indicate no statistically significant difference, and dissimilar superscript letters indicate a significant difference between both age and treatment groups for each mass taken after two-way ANOVA and *post hoc* comparison (P<0.05). Data are presented as means±s.d.

chorioallantoic blood vessel positions. The eggshell was cleaned with 70% isopropyl alcohol and a small hole was made in the shell using a dental drill (Healthco Dental Engine). The inner eggshell membrane was carefully pierced with a sterile 18G needle without damaging any major chorioallantoic blood vessels. A 36-gauge copper constant thermocouple was placed just under the shell on the animal's skin and secured in place with dental wax (Kerr, Jicin, Czech Republic). The T_b of hatchlings was measured in the cloaca. A silicone-coated thermocouple was placed in the cloaca and secured with a small plastic disc glued to the feathers of the hatchling with Super Glue as in Ricklefs and Williams (2003). Body temperature was measured using AD Instruments temperature pods and recorded with a PowerLab 16SP and LabChart 7 software.

Pulmonary ventilation

Estimates of tidal volumes (V_T) in the thermoneutral zone and during cooling trials were made using a barometric technique as in Sirsat and Dzialowski (2016). Changes in pressure associated with breathing were measured with a spirometer (AD Instruments) connected in-line with each respirometry chamber (Menna and Mortola, 2003). Volume calibration was conducted in each respirometry chamber after each trial by injecting known volumes of air (V_{cal}) into the system with a Hamilton syringe (Hamilton, Reno, NV, USA). The corresponding change in pressure (P_{cal}) was used to calibrate the system [K=V_{cal}/P_{cal} as in Szdzuy and Mortola (2008)]. The respirometry chamber's relative humidity was measured using a humidity sensor (HIH 4021, Honeywell, Minneapolis, MN, USA) included to estimate water vapor pressure in the chamber. Tidal volume estimates were calculated from K, chamber T_a and T_b, water vapor pressure and measured pressure changes. Breathing frequency (f, breaths min^{-1}) was calculated from the ventilatory pressure waves using the cycle measurement function in LabChart 7 software. Minute ventilation ($\dot{V}_E$, ml min^{-1}) was calculated by multiplying V_T by f.

Blood collection and morphometrics

After measuring metabolic rate and pulmonary ventilation during cooling, animals were anesthetized with isoflurane. Blood (approximately 0.8–1 ml) was collected in a heparinized 1 ml plastic syringe from the atria of the heart by cardiac puncture. Blood was injected into heparinized capillary tubes and centrifuged at 13,700 ***g*** for 4 min (Autocrit Ultra 3, Becton Dickinson, Franklin Lakes, NJ, USA). Hematocrit was calculated by dividing length of plasma to the whole length of blood column in the capillary tube measured via digital caliper (±0.01 mm; Fisher Scientific, Pittsburgh, PA, USA). The remaining blood was immediately transferred to a 1.5 ml plastic vial and centrifuged (model 16K, Bio-Rad, Hercules, CA, USA) at 600 ***g*** for 10 min at 4°C. Aliquots of plasma were stored at −20°C for later plasma T_3 analysis. Animals were then euthanized by decapitation under isoflurane anesthesia before dissection. Wet masses of whole body, yolk, cardiac ventricles and liver were measured (±0.1 mg; E12140, Ohaus Corporation, Pine Brook, NJ, USA). Head to beak length (measured from the posterior end of the supraoccipital bone to the tip of the beak, with the skin intact) and femur length (measured from the femoral head to the condyles) measurements were made using digital calipers.

Plasma T_3 ELISA assay

Plasma total T_3 concentration was measured with an Accu-Bind ELISA Microwells test kit (125-300, Monobind Inc., Lake Forest, CA, USA) using the manufacturer's standard protocol. The assay is based on a competition reaction between the enzyme conjugate and thyroid hormones for a limited number of antibody-combining sites immobilized on the microplate well. All samples were randomly distributed and analysed within three assay kits (Winter et al., 2013).

Statistical analysis

Comparisons of timing of EP and hatching were analysed between T_3, MMI and control groups using Fisher's exact test, followed by

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post hoc analysis by multiple pairwise Fisher's exact tests with Bonferroni correction for multiple comparisons. For the Fisher's exact test, animals were binned into the day of incubation on which they externally pipped or hatched (bins: days 25, 26, 27, 28 or 29 of incubation). Comparisons of fresh wet body mass, yolk mass, organ masses, femur and head to beak lengths were analysed with two-way analysis of variance (ANOVA) with treatment and developmental age (D25, EP and 1 dph) as the two factors. Significant findings from the ANOVA analysis were followed by a Holm–Šidák *post hoc* test for pairwise multiple comparisons. Differences in hematological variables (hematocrit and plasma total T_3) were examined for their significance using a two-way ANOVA with developmental age and treatment as independent factors followed by all pairwise multiple comparison procedures by Holm–Šidák *post hoc* test. Hedges' *g* with bootstrap 95% confidence intervals were calculated for comparisons of plasma T_3 levels to examine the effect size of the response to altering thyroid hormone status at the end of incubation using estimationstats.com (Ho et al., 2019). We assessed response to altering T_3 levels to be either small (0.2), moderate (0.5), large (0.8) or very large (>1.2; Nakagawa and Cuthill, 2007).

A two-way repeated measures analysis of variance (RM-ANOVA) was performed on $\dot{V}_{O_2}$, animal temperature and ventilation data (f, V_T and $\dot{V}_E$) using treatment (control, T_3 and MMI) and T_a as independent factors at each developmental age (D25, EP and 1 dph). The T_a during the gradual cooling was the repeated factor. To examine changes in basal $\dot{V}_{O_2}$ within the thermal neutral zone (TNZ), we analysed resting $\dot{V}_{O_2}$ at 35°C as whole animal $\dot{V}_{O_2}$ tested with two-way ANOVA with developmental age and treatment as independent factors followed by Holm–Šidák *post hoc* test. T_b and ventilation parameters (f, V_T and $\dot{V}_E$) were examined in the TNZ (T_a of 35°C) across age and treatment using two-way ANOVA followed by Holm–Šidák *post hoc* test for multiple comparisons.

All values are presented as means±s.d. Sample sizes varied for each variable measured across treatments and ages and are provided in the table or figure captions in which those data are presented. The level of significance was set at $P<0.05$. Statistical analyses were conducted using SigmaPlot 12 (Systat Software, Inc., San Jose, CA), SPSS (version 22; IBM, Armonk, NY, USA) and GraphPad Prism 7 software (GraphPad, La Jolla, CA, USA).

RESULTS

Plasma T_3

Thyroid manipulations resulted in significantly altered developmental trajectories of plasma T_3 between the three treatments ($F_{2,53}$=58.22, P<0.001; Fig. 1). There was a significant interaction of age versus treatment effect on plasma T_3 levels ($F_{4,53}$=11.41, P<0.001). A day after dosing on D25, T_3 embryos exhibited elevated total plasma T_3 levels compared with control embryos [Hedges' g=2.37; 95.0% confidence interval (CI) 1.7, 3.17]. MMI treatment had no effect on total plasma T_3 levels in D25 embryos when compared with control embryos (Hedges' g=0.20; 95.0% CI −1.62, 1.65). During the EP stage, embryos supplemented with T_3 maintained a large effect on plasma T_3 compared with controls (Hedges' g=1.39; 95.0% CI 0.09, 3.14). There was a very large effect of MMI on plasma T_3 in EP embryos compared with control EP embryo (Hedges' g=−3.38; 95.0% CI −5.47, −2.4) due to the increase in control plasma T_3 levels. On 1 dph, total plasma T_3 levels of T_3-supplemented hatchlings were similar to those of control hatchlings (Hedges' g=−0.50; 95.0% CI −1.79, 0.87). MMI hatchlings maintained depressed plasma T_3 levels (Hedges' g=−2.91; 95.0% CI −5.77, −1.4) compared with control hatchlings.

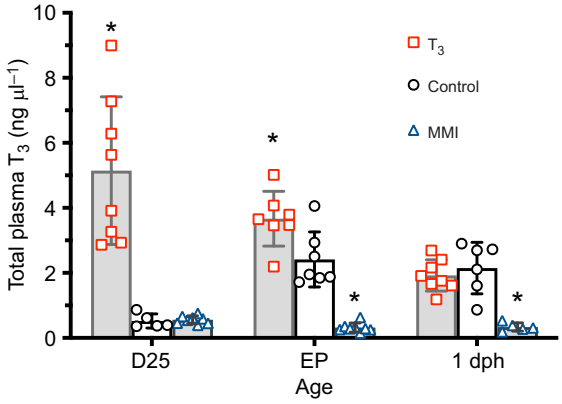

Fig. 1. Total plasma T_3 levels across age and treatment groups of Pekin duck (*Anas platyrhynchos domestica*). Within each age group, an asterisk denotes a statistically significant difference between treatment and control plasma after *post hoc* comparison (P<0.05) and a large effect size (Hedges' g>1). Sample sizes for day 25 embryos (D25), external pipping (EP) and 1 day post-hatching (dph) are: T_3, 8, 7, 8; control, 5, 7, 6; MMI, 8, 8, 5. Data are presented as means±s.d.

Hatching success and developmental progress

Manipulation of T_3 levels on D24 of incubation had a significant effect on the time to reach EP and hatching (Fig. 2; Fisher's exact test, P<0.001). Supplementing T_3 on D24 of incubation resulted in earlier EP and hatching when compared with control eggs. Exposure to MMI on D24 of incubation slowed the timing of EP and hatching when compared with control eggs.

Morphometry

Altering the timing of the increase in plasma T_3 levels had little effect on the morphology of the developing duck (Table 1). Although there were predictable changes occurring with age in all treatments, there were no differences between treatments in yolk-free body mass, yolk sac mass, right femur length or head to beak length within any age. The only morphological measures with a significant effect of treatment were cardiac ventricle mass ($F_{2,90}$=3.58, P<0.05; Table 1) at all ages and liver mass during EP ($F_{2,89}$=8.92, P<0.001). Cardiac ventricle mass of T_3-treated animals was significantly larger than MMI-treated animals at each age examined (P<0.05), but not different from controls (P=0.14). There was no difference in cardiac ventricle mass of control and

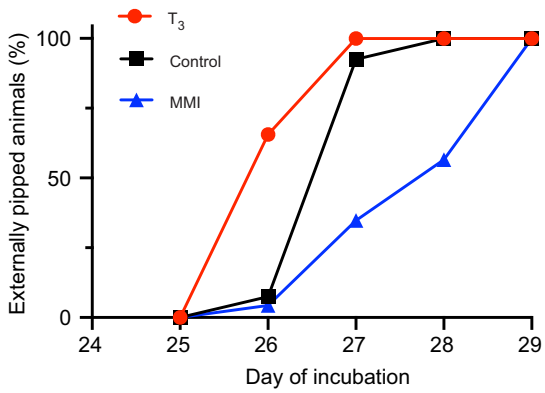

Fig. 2. Percentage of Pekin duck embryos reaching the externally pipped stage in control, T_3 and MMI treatments. External pipping was reached significantly earlier in T_3 animals (N=32) and later in MMI animals (N=23) compared with control animals (N=40) after Fisher's exact test (P<0.05).

MMI groups through development from D25 to 1 dph (P=0.29). MMI animals had the largest liver masses during EP compared with both control and T_3 animals (P<0.01). There was no difference in liver mass between control and T_3 animals throughout development (P=0.5). Hematocrit showed a treatment effect across development ($F_{2,81}$=6.48, P<0.01). Duck embryos and hatchlings of MMI group had a lower hematocrit when compared with both control and T_3 animals (P<0.05) throughout development.

Metabolic rate and body temperature

There was a significant interaction between age and treatment on resting metabolic rates ($\dot{V}_{O_2}$, ml min^{-1}) in the TNZ of 35°C ($F_{4,50}$=5.08, P=0.002; Table 2). T_3-supplemented animals had higher $\dot{V}_{O_2}$ and MMI-treated animals had lower $\dot{V}_{O_2}$ compared with control animals at all ages. On D25 of incubation, MMI-treated embryos had significantly lower $\dot{V}_{O_2}$ than either the T_3 or control embryos. By the EP stage, $\dot{V}_{O_2}$ of T_3-supplemented animals was significantly higher than control animals (P<0.05). In 1 dph hatchlings, the control and T_3-supplemented animals had similar $\dot{V}_{O_2}$, while MMI-treated animals continued to exhibit significantly depressed $\dot{V}_{O_2}$.

To study the development of metabolic endothermic capacity, T_a was gradually lowered and $\dot{V}_{O_2}$ responses were recorded (Fig. 3). Endothermic capacity, defined here as an animal's ability to increase $\dot{V}_{O_2}$ in response to environmental cooling, was only present after hatching. At age D25, T_3-supplemented embryos had a significantly higher $\dot{V}_{O_2}$ (Fig. 3A) during the entire cooling exposure compared with control and MMI-treated embryos (P<0.01). On D25, T_3-supplemented, control and MMI-treated embryos maintained $\dot{V}_{O_2}$ in response to cooling until 27, 27 and 31°C, respectively, at which point, $\dot{V}_{O_2}$ began to drop below resting levels. During the EP stage of development, $\dot{V}_{O_2}$ (Fig. 3B) of T_3-supplemented embryos was significantly higher (P<0.05) and $\dot{V}_{O_2}$ of MMI-treated embryos was significantly lower (P<0.05) compared with control embryos during the entire cooling exposure. T_3-supplemented, control and MMI-treated groups maintained a constant $\dot{V}_{O_2}$ in response to cooling until T_a of 24, 27 and 22°C, respectively, when $\dot{V}_{O_2}$ levels began to decrease. At 1 dph, the control and T_3-supplemented hatchlings exhibited similar significant endothermic responses to cooling, as revealed by an increase in $\dot{V}_{O_2}$ (Fig. 3C). The $\dot{V}_{O_2}$ in MMI-treated hatchlings was significantly lower (P<0.05) compared with control hatchlings during the entire cooling exposure, and never increased significantly in MMI hatchlings when compared with $\dot{V}_{O_2}$ levels at 35°C.

The EP egg T_s and hatchling cloacal T_b were significantly different across age ($F_{2,47}$=3.692, P<0.05) and treatment ($F_{2,47}$=8.398, P<0.001) but their interaction was not significant ($F_{4,47}$=2.049, P=0.103) at TNZ of 35°C (Fig. 3D–F). When considering age as a factor, EP egg T_s was higher than 1 dph cloacal temperature and significantly higher than D25 egg T_s (P<0.05).

Table 2. Whole-animal oxygen consumption (ml O_2 min^{-1}) at 35°C air temperature in control, T_3-supplemented and MMI-treated day 25, externally pipping and 1 day post-hatch Pekin ducks

Age	Control	T_3	MMI
D25	0.46±0.05^{a,b} (6)	0.56±0.07^{a} (6)	0.41±0.05^{b} (6)
EP	0.81±0.11^{c} (11)	1.02±0.16^{d} (6)	0.55±0.11^{e} (5)
1 dph	1.26±0.08^{f} (6)	1.37±0.06^{f} (8)	0.89±0.18^{g} (5)

Sample sizes are provided in parentheses. For each row, similar superscript letters indicate no statistically significant difference after two-way ANOVA and *post hoc* comparison (P>0.05). Data are presented as means±s.d.

When considering treatment as a factor, the MMI-treated group of animals had significantly lower T_s and cloacal T_b than T_3-supplemented and control animals (P<0.005).

Thyroid manipulation had a significant effect on the response to body temperature in EP and 1 dph animals (Fig. 3D–F). At age D25 and EP, T_s of all treatment groups decreased gradually and at the coldest T_a of 22°C, it was more than 15% lower (~33.7°C) than values in the TNZ of 35°C (~40.3°C). At EP, MMI-treated animals had significantly lower egg T_s than controls (P<0.05), but were not significantly lower than T_3 group. After hatching, animals were better able to maintain T_b. Cloacal T_b of 1 dph control and T_3-supplemented hatchlings decreased by only 6% at the lowest T_a of 15°C (~38.7°C) compared with TNZ of 35°C (~40.8°C). The cloacal temperature of MMI-treated 1 dph animals fell by 15% at the coldest T_a (~33.9°C) compared with TNZ of 35°C (~39.6°C). Overall, the 1 dph MMI-treated group had significantly lower cloacal T_b during cooling compared with control and T_3-treated hatchlings (P<0.05).

Development of pulmonary ventilation

Pulmonary ventilation of EP and 1 dph animals displayed different patterns in response to cooling, which was dependent on thyroid treatment (Fig. 4). Within the EP stage, different treatments significantly affected breathing frequency (f) ($F_{2,96}$=12.079, P<0.001; Fig. 4A); MMI-treated embryos had a significantly lower f (P<0.05) than control and T_3-supplemented embryos throughout the gradual cooling. The f of T_3-supplemented embryos was not different from that of the control at T_a of 35°C (P=0.175). f of MMI-treated embryos was significantly depressed (43.9%) compared with control embryos at T_a of 35°C (P<0.001). Upon cooling, f of T_3-supplemented and control embryos decreased gradually, while f of MMI-treated animals was constant initially and then increased at the coldest T_a of 20°C.

At 1 dph, there was no effect of thyroid treatment on f ($F_{2,84}$=2.373, P=0.13; Fig. 4D). At T_a of 35°C, f of T_3-supplemented hatchlings was elevated by 32.7% (P=0.24) and f of MMI-treated hatchlings was 11.8% lower (P=0.146) compared with control hatchlings. The thyroid treatments had a significant effect on f ($F_{2,84}$=2.228, P=0.017; Fig. 4D) in response to gradual cooling. Upon cooling, f of all three treatment groups increased significantly when compared with f at 35°C. The f of MMI-treated hatchlings increased significantly at T_a of 25°C, f of control hatchlings increased significantly at T_a of 20°C, and f of T_3-supplemented hatchlings increased significantly at T_a of 15°C.

The estimated V_T was significantly influenced by treatment within the two age groups (Fig. 4B,E). During the EP stage, treatment had a significant effect on V_T ($F_{2,96}$=14.327, P<0.001; Fig. 4B), T_3-supplemented embryos had significantly higher V_T (P<0.05) and MMI-treated embryos had significantly lower V_T (P<0.05) than control embryos throughout the gradual cooling. Upon cooling, V_T of control embryos increased at T_a of 22°C, but V_T of T_3- and MMI-supplemented hatchings during cooling did not significantly change. In 1 dph hatchlings, treatment had a significant effect on V_T ($F_{2,84}$=13.21, P<0.001; Fig. 4E). V_T of both T_3-supplemented (19.1%, P<0.05) and MMI-treated hatchlings (19.9%, P<0.05) were lower than control hatchlings at T_a of 35°C. Upon cooling, T_3-supplemented and control hatchling V_T began to increase significantly at T_a of 25 and 28°C, respectively. The V_T of MMI-treated hatchlings did not change during the gradual cooling from T_a of 35°C to 15°C.

As expected from responses of f and V_T to T_3 manipulations, $\dot{V}_E$ was significantly affected by thyroid manipulation. Within the EP

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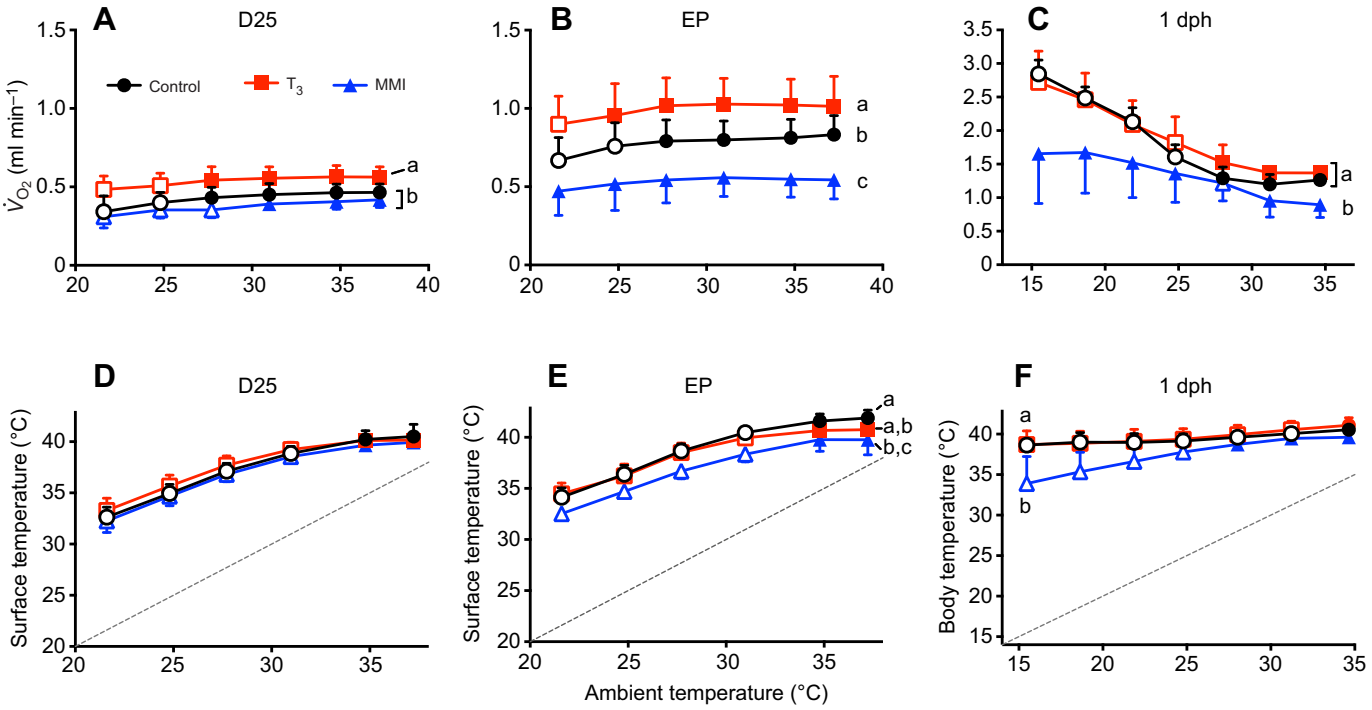

Fig. 3. Whole animal oxygen consumption and egg surface/cloacal temperatures across treatment and age in response to gradual cooling. Pekin duck oxygen consumption ($\dot{V}_{O_2}$) and egg surface temperature (T_s) of control, T_3-supplemented and MMI-treated day 25 (D25) and externally pipped (EP) embryos and 1 day post-hatch (dph) hatchlings in response to cooling at a rate of 9.2°C h^{-1}. (A) $\dot{V}_{O_2}$ response of D25 embryos (control, N=6; T_3, N=6; MMI, N=6). (B) $\dot{V}_{O_2}$ response of EP (control, N=11; T_3, N=6; MMI, N=5). (C) $\dot{V}_{O_2}$ response of 1 dph (control, N=6; T_3, N=8; MMI, N=5). (D) T_s response of D25 (control, N=6; T_3, N=6; MMI, N=6). (E) T_s response of EP (control, N=10; T_3, N=6; MMI, N=6). (F) Cloacal body temperature (T_b) response of 1 dph (control, N=6; T_3, N=7; MMI, N=5). Open symbols denote significant differences (two-way RM-ANOVA and *post hoc* comparison, P<0.05) from the value at an air temperature of 37.5°C for D25 and EP, and 35°C for 1 dph. Treatment groups with different lower case letters show overall significant differences from each other after two-way RM-ANOVA and *post hoc* comparison (P<0.05). Data are presented as means±s.d. The dashed line in D, E and F represents T_a=T_b.

developmental stage ($F_{2,96}$=13.73, P<0.001; Fig. 4C), T_3-supplemented embryos had significantly higher $\dot{V}_E$ (P<0.05) and MMI-treated embryos had significantly lower $\dot{V}_E$ (P<0.05) during gradual cooling. Even though f decreased and V_T increased in T_3-supplemented and control embryos, the $\dot{V}_E$ from EP embryos from these two treatments did not change significantly during gradual cooling.

In 1 dph hatchlings, $\dot{V}_E$ was significantly affected by thyroid manipulation ($F_{1,84}$=19.34, P<0.001; Fig. 4F). T_3-supplemented and control hatchlings had significantly higher $\dot{V}_E$ (P<0.001) than MMI hatchlings at T_a of 35°C and during gradual cooling from T_a of 35°C to 15°C. Upon cooling, $\dot{V}_E$ from all three treatment groups began to increase significantly, beginning with the MMI group at T_a of 32°C, control group at T_a of 28°C and T_3 group at T_a of 25°C. T_3-supplemented and control hatchling $\dot{V}_E$ values were similar throughout the gradual cooling. Because of the lack of increase in f, the $\dot{V}_E$ in MMI-treated hatchlings did not increase to the same extent as control and T_3-supplemented hatchlings.

DISCUSSION

Successful transition from *in ovo* to *ex ovo* life in precocial birds involves maturation of multiple physiological systems leading to increases in aerobic capacity to deliver oxygen to tissues, onset of pulmonary ventilation, and increases in thermogenic capacity to cold challenge (Sirsat et al., 2016). The observations in the current study found that altering the timing of when thyroid hormone T_3 levels increase significantly affects the maturation of oxygen consumption, thermogenesis and pulmonary ventilation during this transition from *in ovo* to *ex ovo* life. Hypothyroid conditions result in alterations in tidal volume, leading to an inability to increase metabolism when exposed to a cold challenge. In addition, the effects of manipulating T_3 levels on various systems resulted in a change in the timing of external pipping and hatching in Pekin duck. These observations support the rise in thyroid hormones occurring at hatching as an important metabolic and developmental regulator for attainment of an endothermic phenotype during the perinatal period in precocial Pekin duck.

Plasma T_3 levels

As with other precocial species, Pekin ducks experience a natural developmental increase in plasma T_3 during hatching (Fig. 1). In control animals, the total plasma T_3 levels increased 3.8-fold from D25 to EP and remained elevated in 1 dph Pekin duck hatchlings. This developmental pattern is similar to developmental changes observed in other precocial species such as chicken, mallard duck, turkey and Japanese quail – low levels throughout embryonic development with a gradual increase to a peak during the peri-hatch period (Christensen et al., 1982; Darras et al., 1992; De Groef et al., 2013; Decuypere et al., 1982; Geris et al., 1998; Lu et al., 2007; McNabb and Hughes, 1983; Moraes et al., 2004; Thomas et al., 1987). The rise of T_3 just before hatching follows a rise in total plasma T_4 (De Groef et al., 2013).

The administration of T_3 and MMI prior to the start of hatching altered how total plasma T_3 levels changed during the perinatal period. The aim of this study was to determine if altering the timing of the increase in plasma T_3 during the peri-hatch period would affect hatching and development of the endothermic metabolic and respiratory phenotype. A single dose of T_3 administered in the air

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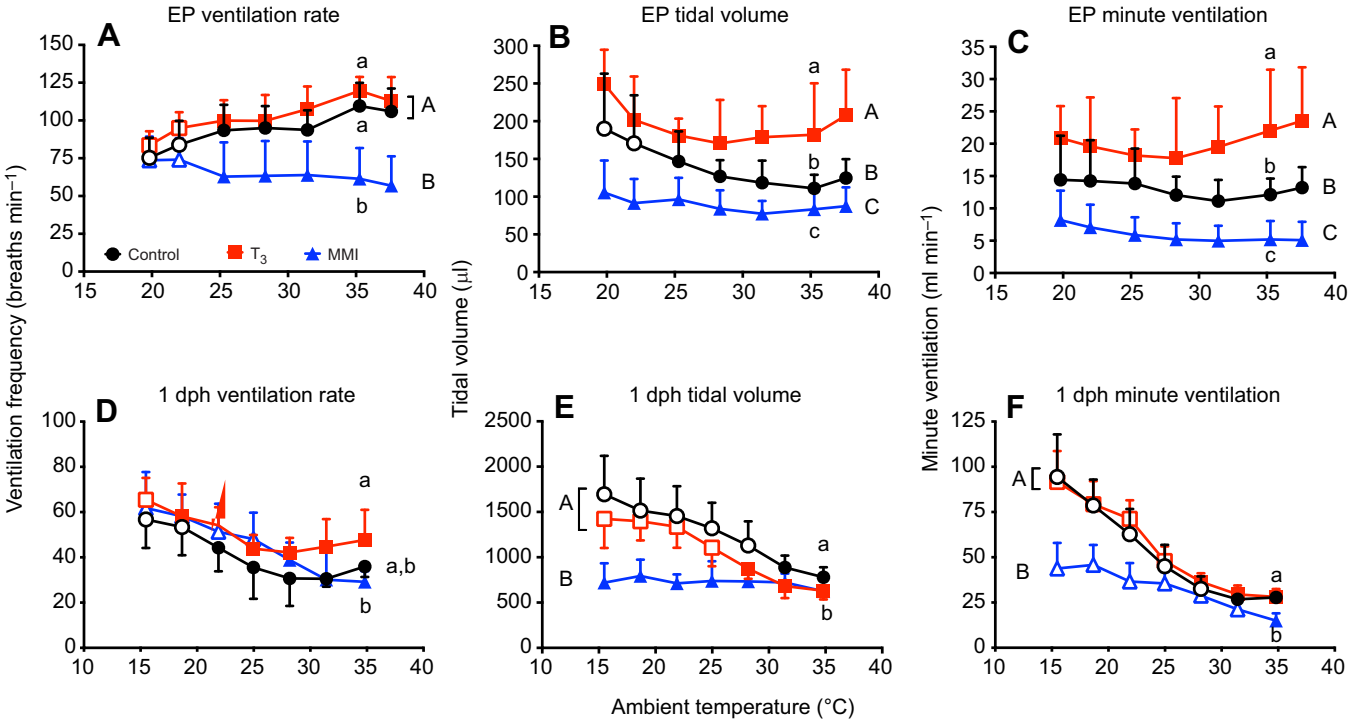

Fig. 4. Pekin duck ventilation rate, tidal volume and minute ventilation during external pipping and 1 day post-hatch across treatment groups: control, T_3 and MMI in response to cooling stress at 9.2°C h^{-1}. (A) Ventilation rate at external pipping (EP) (control, *N*=7; T_3, *N*=6; MMI, *N*=6). (B) Tidal volume at EP (control, *N*=7; T_3, *N*=6; MMI, *N*=6). (C) Minute ventilation at EP (control, *N*=7; T_3, *N*=6; MMI, *N*=6). (D) Ventilation rate at 1 dph (control, *N*=6; T_3, *N*=6; MMI, *N*=5). (E) Tidal volume at 1 dph (control, *N*=6; T_3, *N*=6; MMI, *N*=5). (F) Minute ventilation at 1 dph (control, *N*=6; T_3, *N*=6; MMI, *N*=5). Open symbols denote significant differences within a treatment (two-way RM-ANOVA and *post hoc* comparison, $P<0.05$) from the value at an air temperature of 37.5°C for EP and 35°C for 1 dph. Treatment groups with different lower case letters show ventilation parameters significantly different from each other at 35°C after two-way ANOVA and *post hoc* comparison ($P<0.05$) and different upper case letters show ventilation parameters overall significantly different from each other after two-way RM-ANOVA and *post hoc* comparison ($P<0.05$). Data are presented as means±s.d.

cell produced a significant rise in total plasma T_3 levels above control group values within 24 h of administration on D25, which was maintained for 48 h through the internally and externally pipped stages. By 1 dph, T_3 levels in the manipulated animals had returned to similar levels as the control. A single dose of MMI on D24 decreased total plasma T_3 levels during EP and 1 dph and prevented the T_3 rise typically observed during peri-hatch. In adult chickens, MMI is known to accumulate primarily in the thyroid gland (Van Herck et al., 2013). MMI decreases plasma thyroidal release of T_4 by inhibiting thyroid peroxidase, but did not decrease the release of free T_3 in adult chickens and late-stage chicken embryos (Nagasaka and Hidaka, 1976; Van Herck et al., 2013; Vickers et al., 2012). These findings suggest that plasma T_3 levels are highly regulated during development in birds, just as in mammals (Abdalla and Bianco, 2014; Shepherdley et al., 2002). Although plasma T_4 levels were not measured here, an absence of the peak rise in T_3 in MMI-treated groups suggests that MMI decreased T_4 release from the thyroid gland.

Hatching success and developmental progress

Transitioning from *in ovo* to *ex ovo* life during hatching is a complex orchestration of multiple systems maturing and interacting together. Hatching involves growth and development of the musculus complexus, a hatching muscle, expediting maturation of the respiratory system, retraction of yolk sac, and acceleration of the thermoregulation process in precocial avian species (Karlsson et al., 2015). Supplemental thyroid hormone advanced development as observed by the shortening of hatching by 24 h compared with controls. Accelerated development and shortening of hatching period in response to elevated thyroid hormone levels has been observed in chicken embryos that showed premature yolk sac retraction and early pipping (Balaban and Hill, 1971; De Groef et al., 2013). Similar shortening of the hatching period with T_3 treatment has been observed in non-avian reptiles, snapping turtles and short-necked turtles (Balaban and Hill, 1971; De Groef et al., 2013; McGlashan et al., 2016; Oppenheim, 1973), suggesting that thyroid hormones play a role in hatching across not just birds, but all of the class Reptilia.

Shortening of hatching was associated with lower hatching success by 50% in the T_3-treated group. The greatest number of T_3-treated embryos died before or at EP stage (range D24–D26) compared with controls where mortality occurred between EP and hatching (range D27–D28). In precocial turkey and altricial great tit embryos, exposure to elevated thyroid hormone levels resulted in low hatching during the late stages of development (Christensen, 1985; Ruuskanen et al., 2016). Possible cause of mortality in T_3-treated embryos could be due to increased levels of hypoxia and hypercapnia associated with elevated oxygen consumption and limited oxygen conductance of the eggshell (Decuypere et al., 2006). With the limit on oxygen flux across the eggshell, this could lead to the oxygen supply not meeting the elevated metabolic demand of T_3-treated embryos. Another factor could be a reduction of stored glycogen in the liver along with rising blood glucose utilization due to elevated metabolism, resulting in rapid depletion of glucose sources in the embryo (Christensen et al., 1991).

Unlike accelerated development observed when increasing T_3 earlier in development, MMI-treated embryos delayed hatching

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when compared with control animals (Fig. 2). In chicken embryos, development of the hatching muscle, musculus complexus, has been shown to be delayed or blunted by the thyroid inhibitor thiourea (Brandstetter et al., 1962). Hypothyroidism in chicken embryos during the late stage of development retarded development of cerebral and cerebellar structures along with delayed or an absence of hatching (De Groef et al., 2013; Haba et al., 2011; Karlsson et al., 2015; Roelens et al., 2005). Together, the low $\dot{V}_{O_2}$, ventilatory control and slowed development of the nervous system could delay developmental progress towards hatching in hypothyroid late stage embryos. Overall, the MMI-treated embryos remained in the egg for a longer duration and in some cases needed help out of the eggshell at the EP stage. It seems that the slowed hatching muscle development due to delayed maturation and function of the nervous system may have rendered the perinates too weak to complete the pipping process (Akhlaghi et al., 2012; Haba et al., 2011; Van Herck et al., 2013).

Morphometry

There was a surprising lack of influence of thyroid status on the morphology of the developing Pekin duck. Others have found that hypothyroid conditions as embryos or hatchlings resulted in decreased body mass during hypothyroidism, as seen in precocial chicken embryo, Barnacle geese, altricial nestling zebra finch and adult mallard duck (Deaton et al., 1998; Haba et al., 2011; McNabb, 2006; Rainwater et al., 2008). Rey et al. (2010) reported slower growth of Muscovy ducklings with prolonged treatment of 6-n-propyl-2-thiouracil, another thyroid-peroxidase inhibitor similar to MMI. In the current study, hypothyroidism did not alter structural size, seen in femur length and head to beak length, or mass. This is in contrast to altricial zebra finch nestlings where hypothyroidism resulted in decreased tibiotarsal length and delayed nervous system development in chicken embryos (Haba et al., 2011; McNabb, 2006; Rainwater et al., 2008).

One of the only morphological traits to show an effect of thyroid treatment was the heart. The hearts of hatchlings supplemented with T_3 were larger than those treated with MMI. Cardiovascular system development and maturation is important for hatching and post-hatch life and is one of the limiting factors for maximal aerobic metabolism in post-hatch life of endotherms (Hillman et al., 2013). Twenty-four hour exposure to elevated plasma T_3 during the late stage of incubation manifested in increase in cardiac mass, resting heart rate (f_H), intrinsic mean arterial pressure (Pa_{mean}) and heart rate, increased cholinergic tone on f_H and blunted α-adrenergic response to f_H and Pa_{mean} in Pekin duck embryos (Sirsat et al., 2018). T_3 has been documented to have both direct and indirect effects on the cardiovascular system such as increased cardiac mass, heart rate and cardiac contractility, thereby increasing stroke volume and cardiac output, decreasing peripheral resistance, and increasing blood volume and red blood cell number (Klein and Ojamaa, 2001). Concomitant rise of plasma T_3 levels during hatching and attainment of endothermy play crucial roles in the increase in size and function of the cardiovascular system to support gaseous and nutrient delivery to the rest of the body.

Whole-animal metabolic rate and body temperature

The active form of thyroid hormone, T_3, is known to affect resting metabolism and oxygen consumption (Barker, 1951; Goglia, 2015; Welcker et al., 2013). Our finding confirms that early supplementation of T_3 increased resting $\dot{V}_{O_2}$ significantly in D25 and EP animals compared with controls. By 1 dph, the $\dot{V}_{O_2}$ of T_3-supplemented animals, which hatched a day early, was similar to that of control hatchlings. This finding may be explained by the decrease in total plasma T_3 values by 1 dph T_3 hatchlings and the control animals catching up developmentally with the T_3-supplemented hatchlings. However, hypothyroidism induced by MMI resulted in lower $\dot{V}_{O_2}$ in EP and 1 dph animals compared with controls. On further applying cooling stress to assess development of endothermic responses, patterns of elevated $\dot{V}_{O_2}$ in T_3-supplemented and depressed $\dot{V}_{O_2}$ in MMI-treated animals were observed. These findings of resting $\dot{V}_{O_2}$ and its response during cold challenge in both treatment groups suggest that $\dot{V}_{O_2}$ values correlate with total plasma T_3 values in ducklings. Similar observations were reported in mammals, birds and fish (Barker, 1951; Bishop et al., 1995; Hulbert and Else, 2004; Little et al., 2013; Lynch et al., 1985). Thyroid hormones exert a positive calorigenic effect on the tissue that increases the demand for oxygen. Thyroid hormone is required in nearly all tissues, with major effects on oxygen consumption and metabolic rate (Ma et al., 2004). At the tissue level, thyroid hormone increases $\dot{V}_{O_2}$ in chicken myotubes *in vitro*, and cardiac ventricles, liver and skeletal muscle in tufted duck (Bishop et al., 1995; Bobek et al., 1977).

Similar to $\dot{V}_{O_2}$, body-surface and cloacal temperature of MMI-treated animals were significantly lower during cooling stress at EP and 1 dph. Body-surface and cloacal temperature of T_3-treated animals were not different from control at all three stages. Hypothyroidism in MMI-treated animals significantly affected response to cold at EP and 1 dph stage. This observation supports previous findings in mammals, birds and fish that thyroid hormone is crucial for cold acclimation (Hulbert and Else, 2000; Little et al., 2013).

This study confirms the role of thyroid hormones, specifically T_3, affecting the development of resting systemic metabolism and body temperature regulation and attainment of endothermic phenotype. The findings support observations from previous studies that plasma thyroid hormone levels correlate with development of endothermy and thermoregulation capacity (Decuypere et al., 2005; Freeman, 1971; Klandorf et al., 1981). Within metabolically active tissue (i.e. skeletal muscle, heart and liver), thyroid hormone acts via thyroid hormone receptors, which induce expression of metabolically important genes, interact with key cellular metabolic regulators and result in regulation of mitochondrial capacity and metabolic flux (Hulbert, 2000; Little and Seebacher, 2014). In mammals, at the cellular level, thyroid hormone induces changes in fatty acyl composition of phospholipid membrane, stimulating cellular Na^+–K^+-ATPase and SERCA activity, leading to increased ATP use (Harper and Seifert, 2008; Mullur et al., 2014). Thyroid hormones also induce mitochondrial uncoupling by promoting increased proton leak and heat production in mammals (Harper and Seifert, 2008; Hulbert, 2000). All these activities increase tissue oxygen consumption, particularly in skeletal muscle. In birds, elevation of thyroid hormones increases avian *UCP* gene expression and heat production during cold acclimation (Collin et al., 2003). In birds, shivering thermogenesis is the major source of heat production in response to a cold challenge compared with non-shivering thermogenesis via brown adipose tissue in mammals (McNabb, 2006). In the central nervous system, thyroid hormone has a significant effect on hypothalamus, indirectly modulating sympathetic outflow signaling and thereby β-adrenergic receptor-activated heat production in brown adipose tissue (mammals) and skeletal muscle (mainly in birds), and an increase in heart rate and cardiac output (Little and Seebacher, 2014; McNabb, 2007). All these effects contribute to changes in resting metabolic rate associated with changes in thyroid hormone levels. The local

hypothalamic functions of thyroid hormone during development of endothermy and thermoregulation in birds are yet to be examined. We still do not know the answers to the questions of how thyroid hormone increases skeletal thermogenesis using central pathways and how central and peripheral tissue thermal homeostasis integrate, develop and mature during the peri-hatch period.

Pulmonary ventilation

The current study reported significant effects of altering plasma T_3 levels at hatching on pulmonary development in the hatching duckling. This is the first study to examine the contribution of T_3 to the development of ventilation, simultaneously with endothermic development, in precocial birds. In general, T_3- and MMI-treated EP embryos had elevated or decreased f, V_T and $\dot{V}_E$, respectively, when compared with controls in the TNZ and during gradual cooling. Lower thyroid hormone levels during hatching resulted in a developmental decrease of V_T and $\dot{V}_E$ in the TNZ and the response to cooling compared with control (Fig. 3). The dramatic effects of hypothyroidism could be explained by the regulatory role T_3 plays in development and maturation of the avian respiratory system. During internal pipping, the avian embryo undergoes transition from chorioallantoic to pulmonary respiration. During this transition, fluid-filled lungs undergo fluid resorption, production of surfactant, expansion of respiratory ducts and increase in pulmonary circulation (Barker et al., 1990). In the mammalian fetus, thyroid hormones sensitize pulmonary fluid resorption to catecholamines, increase expression of pulmonary β-adrenergic receptors, and increase activity of Na^+–K^+-ATPase (Barker et al., 1990; Das et al., 1984; Wilson et al., 2007). In chicken embryos, thyroid hormone β-receptor (TR-β) mRNA expression in lung tissue increases towards the end of incubation (Blacker et al., 2004). In sauropsids like chicken embryos, thyroid hormones regulate surfactant phospholipid production and maturation along with glucocorticoids (Sullivan and Orgeig, 2001). During development of the chicken lung, thyroid hormones also affect blood flow through changes in pulmonary vascular resistance via action on kinin–kallikrein and angiotensin converting enzyme systems towards the end of incubation (Decuypere et al., 1990). Hypothyroid treatment in chicken embryos resulted in solid lung tissue on D19, decreased breathing rate prior to hatching, increased TR-β expression, and attenuation of an increase in angiotensin converting enzyme activity (Bjørnstad et al., 2016; Burton and Tullett, 1985; Wittmann et al., 1987). Hypothyroidism in adult mammals decreased central respiratory drive along with reduction in respiratory muscle strength (Milla and Zirbes, 2012). Such central nervous system and respiratory muscle effects of altered thyroidal status on developing birds have not yet been examined. Thyroid hormone effects on avian pulmonary fluid reabsorption, surfactant production-maturation, pulmonary circulation and central respiratory control play critical roles in maturation of the respiratory system.

Conclusion

Hatching and development of endothermy in precocial birds are complex processes, limited by many factors, many of which are directly affected by thyroid hormones. In the current study, we provide evidence that altering the timing and level of plasma T_3 alters development of metabolism, body temperature regulation and pulmonary ventilation at various stages of hatching in a precocial bird. The goal of this study was to observe the effects of altering T_3 levels within physiological limits on the development of metabolic pathways associated with attaining endothermy. This study supports the role of thyroid hormones in regulating endothermic development and transitioning to post-hatch life. Accelerating the increase in plasma T_3 resulted in accelerated development of hatching and attaining an endothermic phenotype. Hypothyroid conditions during hatching resulted in altered ventilatory function and endothermic-associated metabolism. Metabolism and pulmonary ventilation correlated with total plasma T_3 values at EP and 1 dph. Pulmonary ventilation requires the elevated T_3 levels at hatching to ensure proper development. Under the hypothyroid treatment, the hatchling was unable to increase $\dot{V}_E$ due to an invariable tidal volume. Even though the current study elucidates thyroid hormone effects on $\dot{V}_{O_2}$, body temperature and pulmonary ventilation, we still do not fully understand how thyroid hormone augments thermoregulation control in the hypothalamus and respiratory drive at the central level in developing vertebrate embryos. A recent study showed the role of thyroid hormones in ventromedial hypothalamus which maintains thermoregulation mainly by modulating sympathetic signaling to heart, skeletal muscle, liver and brown adipose tissue in adult mammals (Lage et al., 2016). Very little information is available about how thyroid hormones and central nervous system especially hypothalamic thermoregulatory homeostasis interact and mature and how they contribute to transition, hatching and cold adaptation in precocious hatchlings.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.S.S., E.M.D.; Methodology: T.S.S., E.M.D.; Formal analysis: T.S.S., E.M.D.; Investigation: T.S.S., E.M.D.; Resources: E.M.D.; Data curation: T.S.S.; Writing - original draft: T.S.S., E.M.D.; Writing - review & editing: T.S.S., E.M.D.; Supervision: E.M.D.; Funding acquisition: E.M.D.

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