Original Research Paper

Validating a Novel Model of Induced-Thyrotoxicosis in White Leghorn Chicken

Holly Vopal[1,*], Tyler Hill[1], Andrea Dwiggins[1], Carson Corrick[1], Hannah Ankrom[1], Peri Gray[1], Kirsten Bogunovich[1], Lindsey Morais[1], Haley Carter[1], and Daron Weekley[1]

[1]Department of Biological Sciences, West Liberty University, West Liberty, USA.

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**Corresponding Author*: Holly Vopal, West Liberty University, West Liberty, USA;
Email: holly.racine@westliberty.edu

Abstract: Thyroid hormones play a critical role in growth and maintenance in all organ systems. In pregnant women, excess thyroid hormones in circulation, or thyrotoxicosis, has been associated with various fetal abnormalities including craniosynostosis (CS), the premature fusion of the cranial sutures. The mechanism of thyrotoxicosis-induced CS is elusive and therefore requires further investigation. To enhance current knowledge and uncover this indefinable mechanism, we have proposed a new model of induced-thyrotoxicosis mimicking maternal thyrotoxicosis using White Leghorn chicken embryos exposed to a low dose of 25 ng thyroxine (T_4) on developmental days 11 and 15. While our overall objective was to establish this model to study the effects of maternal thyrotoxicosis in the development of CS in utero, this model may be effective for studying other developmental conditions related to increased circulating thyroid hormones during embryonic development. Our model maintained over 90% survivability while inducing morphological and molecular changes in target tissue types, including bone. We confirmed thyrotoxicosis in the chicken embryos by quantifying levels of T_4 in serum by ELISA and observed attenuated body growth with thyroxine exposure supported by a 20% decrease in body mass ($p<0.001$), 5% decrease in body length ($p<0.001$), 20% decrease in third metatarsal length ($p<0.01$), and 7% decrease in tibiotarsal length ($p<0.01$). Our results support that our proposed model of induced-thyrotoxicosis in developing chicken embryos will be efficient for studying the effects of increased circulating thyroid hormones on the development of multiple organ systems.

Keywords: bone development; craniosynostosis; liver; thyrotoxicosis; thyroxine

Introduction

In the developing animal and human, thyroid hormones (THs) regulate growth and metabolism in every cell of the body. The production of THs is regulated by the hypothalamic-pituitary axis. Thyrotropin-releasing hormone (TRH) from the hypothalamus stimulates the production of thyroid-stimulating hormone (TSH) in the anterior pituitary, which target the thyroid gland to release THs which include thyroxine (T_4) and triiodothyronine (T_3). While T_4 makes up the majority of the TH secreted, it is deionized by deiodinase enzymes to form the biologically active T_3 in peripheral tissues (Bassett & Williams, 2016; Bruinstroop et al., 2023; Leitch et al., 2020; Peeters & Visser, 2000). Tissue development and differentiation is affected by THs in the body's major organ systems. Increased levels of TH concentrations, or thyrotoxicosis, can therefore alter normal tissue development. During pregnancy,

mothers can also expose the fetus to increased THs in cases such as maternal hyperthyroidism. Maternal hyperthyroidism occurs in approximately 0.1-1.0% of pregnant women, mostly due to Grave's disease which has been raising in prevalence in the United States (Nygaard, 2015; Barajas Galindo *et al*., 2022). Augmented THs during fetal development has been reported to increase the risk for infants developing craniosynostosis (CS), the premature fusion of one or more cranial sutures (Rasmussen *et al.*, 2007; Cray *et al*., 2013). Studies have described anywhere from 3.5% to 4.4% of reported cases of CS to be linked to mothers with recognized thyroid dysfunction (Cray *et al.*, 2013; Carmichael *et al*., 2015). In a 2015 study, it was concluded that even mothers that displayed risk factors associated with thyrotoxicosis, were more at risk for CS (Carmichael *et al*., 2015). As a condition of complex etiology, the mechanisms of thyroid-related CS during development are still obscure.

Multiple research efforts have been made to delineate the pathogenesis of thyroid-related craniofacial anomalies during development. However, in these studies, only rodent models are described (Akita *et al*., 1996; Cray *et al*., 2013; Howie *et al*., 2016) . An alternative model of study would be an avian model of induced thyrotoxicosis using fertilized chicken eggs. There are multiple benefits to using chicken embryos as a model organism. Specifically, fertilized chicken eggs can be used to mimic maternal hyperthyroidism in a controlled environment, as it can be manipulated without the need of the mother. Additionally, the chicken embryo develops and hatches in 20-21 days providing a reasonable time frame for completion of studies. In regard to cranial development, the chicken embryo has been useful as a comparative model because there are similarities between avian and human craniofacial development. For instance, the embryonic tissues that give rise to the cranial sutures and bones, such as the neural crest and mesoderm, are highly conserved between avians and humans (Schock *et al*., 2016).

While studies have not specially focused on using chicken embryos in the investigation of the molecular pathways associated with thyrotoxicosis-induced CS, the chicken has been utilized in other studies related to abnormal thyroid development. The developing chicken relies on the maternal supply of THs until mid-gestation similar to human fetal development (Darras, 2019; Prati et al., 1992). Both maternal and exogenous THs play a role in many key signaling pathways that regulate the development of organ systems including the skeletal system, biliary system, and cardiovascular system. Increased THs in circulation have been reported to impact how the tissues in these systems develop and function.

Collectively, we developed an avian model of induced-thyrotoxicosis for the investigation of thyroid-induced developmental abnormalities. Our avian model utilizes White Leghorn chicken embryos exposed to thyroxine (T_4) following the development of the thyroid gland and onset of skull ossification (Beckett, 2016; Darras, 2019). Our model maintains over 90% survivability while inducing morphological and molecular changes in target tissue types characteristic of augmented TH activity. This model is intended to be used in imminent studies to delineate the molecular cause of thyroid-related mechanisms involved in the pathophysiology of CS conditions that arise during fetal development.

Materials and Methods

Animals

Fertilized, Premium Specific-Pathogen Free (SPF), White Leghorn (*Gallus gallus domesticus*) chicken eggs purchased from Charles River Laboratories (North Franklin, CT, USA) were used in the present study. Fertilized eggs were incubated at 99.8ºF and 60-65% relative humidity and were turned automatically six times a day (Hova-Bator 2370). Eggs were candled and viability was recorded daily. Non-fertile eggs were excluded at 5 days post fertilization (dpf) and therefore were not included in the study. On 19 dpf, chicken embryos were isolated, body mass was recorded and tissue samples were collected. All procedures with chicken embryos were approved by the Animal Care and Use Committee of West Liberty University.

Experimental Procedure

Both thyroid hormones were purchased from Sigma Aldrich. The 3,3',5-triiodo-L-thyronine (T_3) stock solution (100 µg/mL) was prepared in 0.1 N NaOH and then diluted with 0.9% NaCl to the appropriate dose. L-Thyroxine (T_4) stock solution (100 µg/mL) was prepared in 0.1 N NaOH and then diluted with 0.9% NaCl to the appropriate dose. On the day of injections, 1 µL of the thyroid hormone treatment solution was injected beneath the chorioallantoic membrane into the air cell of the

fertilized chicken egg. In the effort to establish an effective dosage, the following total doses were administered: (1) 0.5 µg T_3 + 1.0 µg T_4, (2) 0.25 µg T_3 + 0.5 µg T_4, (3) 0.125 µg T_3 + 0.25 µg T_4, (4) 1.0 µg T_4, (5) 0.5 µg T_4, (6) 50 ng T_4, (7) 5.0 ng T_4, (8) 0.5 ng T_4, and (9) 50 pg T_4. Multiple injections were given at various junctures between 12 dpf to 18 dpf to obtain the reported treatment dosage. However, following these preliminary studies, the established treatment regimen was 25 ng T_4 injected 11 dpf and 15 dpf for a total dose of 50 ng T_4 (N=26). Days 11 and 15 dpf were chosen since the thyroid gland is developed and thyroid hormone secretion is actively stimulated by the adenohypophysis. Additional, the timing between injections allows for uniformly spaced treatment days prior to collection. Contiguous with the thyroid hormone treatments, 1 µL of saline (1:3 of 0.1 N NaOH:0.9% NaCl) was also administered into a control group of fertilized chicken eggs (N=25).

Serum collection and quantification of TSH and T_4 by ELISA

Blood was collected from the chorioallantoic vessels of chicken embryos collected at 19 dpf. If vessels were unattainable, collection was performed by cardiac puncture. Blood was allowed to clot at 4ºC overnight before samples were centrifuged for 20 minutes (1000 x g) to isolate serum. Serum was stored at -20ºC until used for ELISA to quantify levels of chicken thyroid stimulating hormone (TSH) and thyroxine (T_4). ELISA kits were purchased from MyBioSource (San Diego, CA, USA). An enzyme-linked immunosorbent assay (ELISA) was performed as described by the provided kit protocol. Assay sensitivity was 46.875 pg/mL for TSH and 1.29 ng/mL for T_4. The intra- and inter-assay coefficients of variation for TSH and T_4 were 8.4% and 7.3%, and 6.5% and 5.3%, respectively.

Measurement of body length, body mass, digit length, and tibiotarsus length

Upon collection at 19 dpf, chicken embryos were measured in length (cm) from beak tip to the distal end of the third digit. Additionally, the third digit was measured from the distal tarsometatarsus to the distal phalanx to determine average metatarsal length. Embryos were then weighed on a laboratory scale to determine body mass (g). The tibiotarsus was dissected, cleaned, and dried overnight. Tibiotarsi were then scanned on a flatbed scanner and length (mm) was measured from calibrated images by drawing a line from the proximal-most tibiotarsus to intercondyloid fossa using ImageJ software (Schneider *et al.*, 2012).

PCR analysis of livers

To extract total RNA, the livers from chicken embryos at 19 dpf were dissected and then homogenized in TRIzol™ reagent (Invitrogen™) with three 3.2 mm steel beads using a bead beating homogenizer (FastPrep-24™, MP Biomedicals). After, chloroform was added to the digested samples and centrifuged at 12,000 g and 4ºC for 15 minutes, the upper aqueous phase was taken to precipitate total RNA using the PureLink™ RNA Mini Kit (Invitrogen™) per manufacturer's instructions. The extracted RNA was treated with DNAase kit (Invitrogen™) to remove genomic DNA contamination. The levels of transcripts were determined by quantitative real-time PCR using the qPCRBIO Probe 1-Step Go Hi-ROX kit (Genesee Scientific) per manufacturer's instructions. The Applied Biosystems™ TaqMan™ gene expression assays (ThermoFisher) for *type I iodothyronine deiodinase* (*DIO1*) and *type III iodothyronine deiodinase* (*DIO3*) were quantified using the Applied Biosystems™ StepOnePlus Real-Time PCR System. Data were normalized to *succinate dehydrogenase* (*SDHA*) expression by ΔCT. Quantitative data were compared for gene expression change due to T_4 exposure by ΔΔCT methodology. True fold change was determined by taking the log2 of $2^{-(\Delta\Delta CT)}$ stating decreased expression as a value less than 0.

Statistics

Data are presented as means ± SD. All statistical analyses for this study were performed using R software (R Core Team) with a *p*-value below 0.05 as a criterion for significance. The distribution of each independent variable was assessed by visual inspection of histograms and Q-Q plots in combination with a Shapiro-Wilk test. Data were normally distributed and met the assumptions of homoscedasticity. Body mass was compared between six treatment doses using an ANOVA. Differences between variables were determined using the Tukey post hoc analysis. For body length, metatarsal length, tibiotarsal length, serum TSH and T_4 concentrations, and *DIO1* and *DIO3* mRNA

expression, the difference between saline and T_4 exposure for each variable was assessed using a Welch two sample *t* test.

Results

Establishing a treatment dose regimen

Survivability, or viability, of fertilized chicken embryos exposed to thyroid hormones and saline was recorded following various dosage regimens. The preliminary experimental design intended for injections to be administered on 12 dpf, 14 dpf, 16 dpf and 18 dpf. However, often the embryos did not survive for these sequential injections. Therefore, survivability is summarized based on the total injection dose that was administered prior to being documented as nonviable (Table 1). Chicken embryos (N=13) that were exposed to 0.5 µg T_3 + 1.0 µg T_4 were reported nonviable by 14 dpf. Reducing the dose by half to 0.25 µg T_3 + 0.5 µg T_4 also lead to death by 14 dpf (N=7). Another fifty percent reduction to 0.125 µg T_3 + 0.25 µg T_4 continued to lead to death by 15 dpf (N=6). All chicken embryos exposed to saline (N=7) maintained viability by 19 dpf. After eliminating T_3 from injection solution, survivability rate increased to 20% and 27% for those exposed to 1.0 µg T_4 and 0.5 µg T_4, respectively.

Table 1. Preliminary efforts to establish dosage resulting in inadequate survivability.

Total Treatment	Number of Individuals	Survivability Rate (%) by 19 dpf
Saline (Control)	7	100%
0.5 µg T_3 + 1.0 µg T_4	13	0%
0.25 µg T_3 + 0.5 µg T_4	7	0%
0.125 µg T_3 + 0.25 µg T_4	6	0%
1.0 µg T_4	30	20%
0.5 µg T_4	11	27%

In efforts to increase survivability rate, chicken embryos were exposed to graded doses of T_4 at 11 dpf and 15 dpf. Survivability rate was determined based on number of viable embryos on 19 dpf. Body mass was also recorded. By 19 dpf chicken embryos exposed to saline (N=9) resulted in a survival rate of 100% and mean body mass of 26.16±1.64 g. Chicken embryos exposed to a total dose of T_4 at 50 pg (N=12), 0.5 ng (N=12), 5.0 ng (N=12), 50 ng (N=12), 0.5 µg (N=10) had a survival rate of 100%, 92%, 100%, 92%, and 30%, respectively. Body mass decreased with increasing dose of T_4 recorded as 25.2±1.99 g, 24.69±2.91 g,23.48±1.89 g, 20.60±4.26 g, 17.38±2.01 g, respectively. Compared to controls, there was a significant reduction in body mass for 50 ng T_4 ($p<0.001$) and 0.5 µg T_4 ($p<0.001$) (Figure 1). It was determined that the most effective treatment regimen that had significantly reduced body mass while still maintaining an acceptable survivability rate above 90%, was a total dose of 50 ng T_4.

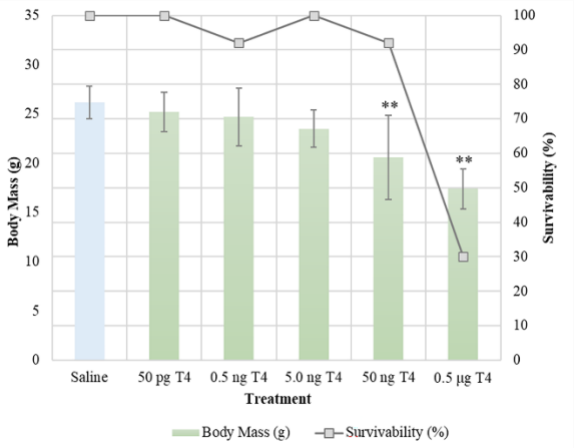

Figure 1. Clustered column graph showing dose-response relationship between treatment and the measured body mass (left y-axis) at 19 dpf. The percent of embryos that survived treatment by 19 dpf (right y-axis) also overlays body mass (line with square marker). While body mass significantly decreased following the 50 ng T_4 (N=12) and 0.5 µg T_4 (N=10) treatment, the survivability of the higher 0.5 µg T_4 treatment dose was only 30%, while 50 ng T_4 maintained a 92% survivability, ideal for continuing with this model in future experiments. Body mass results are represented as the mean ± SD (standard deviation); comparisons were made between the body mass of those embryos receiving the control treatment (saline) and each T_4 treatment; significance was determined using the Welch's two student t-test (***,$p<0.001$).

Confirmation of thyrotoxicosis in circulation

On 11 dpf and 15 dpf, chicken embryos were exposed to 25 ng T_4 introduced into the air cell. To confirm that injection solutions were effectively taken up by the embryonic circulation, serum analysis by ELISA quantified T_4 and TSH in collected blood samples on 19 dpf (Figure 2). The concentration of TSH in circulation was 2555.79±1427.49 pg/mL in control serum samples (N=7), while levels declined to 2012.96±1097.82 pg/mL following T_4 exposure (N=10) ($p=0.3885$). The concentration of T_4 in circulation was 41.61±21.59 ng/mL in control serum samples (N=12), while levels increased to 85.48±37.33 ng/mL following T_4 exposure (N=14) ($p<0.01$).

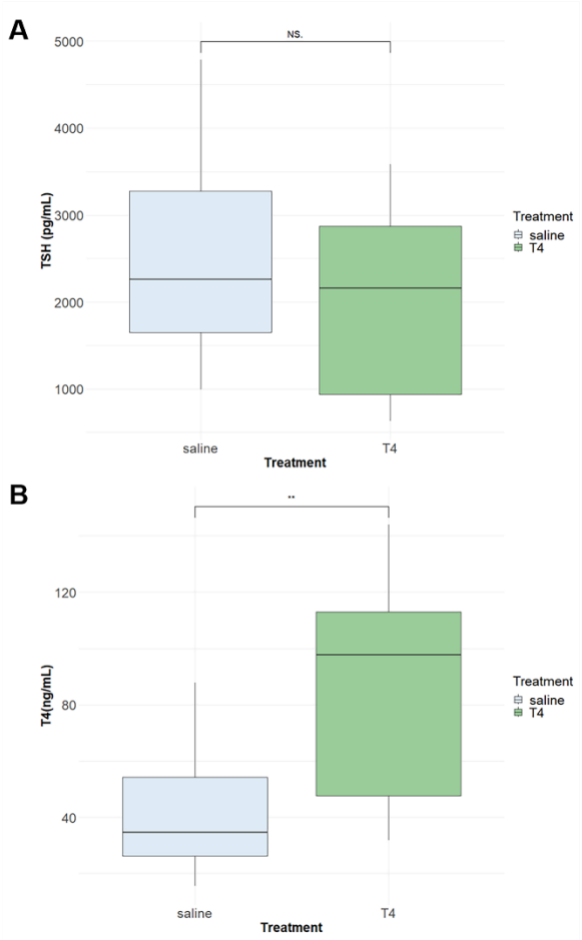

Figure 2. Box plot summarizing the distribution of quantified serum concentrations of (A) TSH and (B) T_4 from chicken embryos on 19 dpf following thyroxine exposure. (A) The concentration of TSH was 2555.79 ± 1427.49 pg/mL (N=7) following saline exposure but declined to 2012.96 ± 1097.82 pg/mL (N=10) following thyroxine exposure (results were not significant). (B) The concentration of T_4 determined by ELISA was 41.61 ± 21.59 ng/mL (N=12) following saline exposure but increased to 85.48 ± 37.33 ng/mL (N=14) following thyroxine exposure ($p<0.01$). The box encompasses the interquartile range of concentrations with a line marking the median and "whiskers" extending from the box to the minimum and maximum values; comparisons were made between the concentrations of those embryos receiving the control treatment (saline) and each T_4 treatment; significance was determined using the Welch's two student t-test (ns, $p>0.05$; **, $p<0.01$).

Metabolism of T_4 in the liver

Gene expression was assessed in dissected livers to evaluate changes in expression of iodothyronine deiodinase enzymes responsive to T_4 in hepatocytes. This analysis was done by quantifying expression levels of *DIO1* and *DIO3* by qRT-PCR (N=7 per treatment). In the comparison of saline to T_4 exposure, there was a 2-fold decrease in *DIO3* mRNA expression following thyroxine exposure ($p<0.01$) while *DIO1* mRNA expression was not affected by thyroxine exposure ($p=0.658$) (Figure 3).

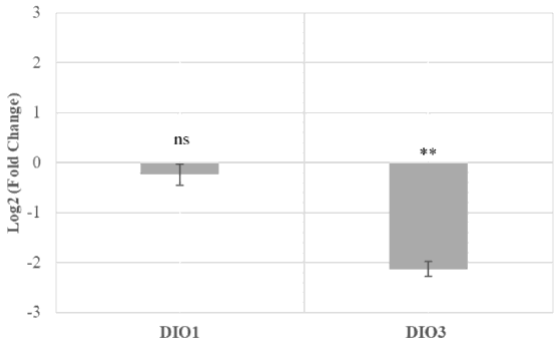

Figure 3. Bar graph showing true fold change of *DIO1* and *DIO3* mRNA expression levels in 19 dpf chicken embryo livers (N=7 per treatment for each target). The calculated log2 fold change for *DIO1* expression following thyroxine exposure was -0.24 (results were not significant). The calculate true fold change for *DIO3* expression following thyroxine exposure was -2.13 ($p<0.01$). These data supported a significant decrease in *DIO3* mRNA expression in livers following thyroxine exposure. Log2 (fold change) results are represented as the mean ± SEM (standard error of the mean); comparisons were made between the calculated ΔCT of those embryos receiving the control treatment (saline) and T_4 treatment; significance was determined using the Welch's two student t-test (ns, $p>0.05$; **, $p<0.01$).

Morphometric analysis following T_4 exposure

Following exposure of 25 ng T_4 on 11 dpf and 15 dpf, attenuation of interstitial growth was observed in chicken embryos by 19 dpf (Figure 4). The measured body length of chicken embryos following saline exposure (N=26) was 15.51±0.61 cm. Following T_4 exposure (N=25), body length was reduced to 14.77±0.88 cm ($p<0.001$). To confirm this observed growth restriction, morphometric analyses were also performed on specific limb structures including toe (metatarsus) length and tibiotarsal length (Figure 5). The length of the third metatarsus of chicken embryos exposed to saline was 16.4±0.32 mm (N=16), while following T_4 exposure declined to 13.1±0.16 mm (N=14) ($p<0.01$). Similarly, the tibiotarsus, a long bone between the femur and tarsometatarsus in chicken embryos was 20.1±1.18 mm following saline exposure (N=15), but measured 18.6±1.42 mm following T_4 exposure (N=14) ($p<0.01$).

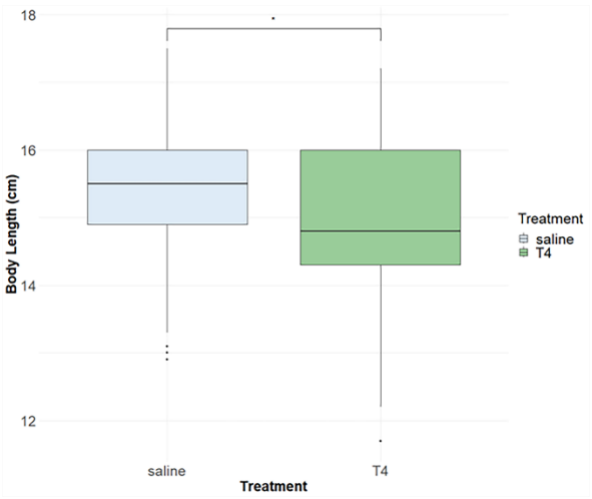

Figure 4. Box plot summarizing the distribution of measured body length of chicken embryos on 19 dpf following thyroxine exposure. Body length following saline exposure was 15.51 ± 0.61 cm (N=26) and decreased to 14.77 ± 0.88 cm (N=25) following thyroxine exposure ($p<0.05$). The box encompasses the interquartile range of concentrations with a line marking the median and "whiskers" extending from the box to the minimum and maximum values; comparisons were made between the measured body lengths of those embryos receiving the control treatment (saline) and each T_4 treatment; significance was determined using the Welch's two student t-test (*, $p<0.01$).

Discussion

In the preliminary efforts to establish a dose for the model of induced-thyrotoxicosis, multiple regimens were tested. The initial experiment exposed fertilized chicken embryos to 0.5 µg T_3 in combination with 1.0 µg T_4 every other day from 4 dpf to 18 dpf as previously described (Darras, 2019). However, by 8 dpf all TH treated embryos were nonviable (data not shown). The succeeding trial in this early phase of experimentation exposed the embryos to the same dosage, but injected later in development at 12 dpf, 14 dpf, 16 dpf and 18 dpf after the thyroid gland is known to be functional and contributing to the regulation of TH levels (Darras, 2019). This adaptation was believed to decrease the risk of death caused by the overwhelming systemic effects with uncontrolled hyperthyroidism (Stagnaro-Green & Pearce, 2012). Despite these alterations to the injection junctures, by 14 dpf all thyroid hormone treated embryos were nonviable. The next attempt to maintain viability was to decrease the dosage while maintaining injection time points to be 12 dpf, 14 dpf, 16 dpf and 18 dpf. From grading the dose of TH exposure from 0.5 µg T_3 + 1.0 µg T_4 to 0.125 µg T_3 + 0.25 µg T_4, there were still no viable embryos by the experimental endpoint at 19 dpf. It is important to note that in all experiments, the embryos exposed to our saline control solution maintained 100% viability up to 19 dpf.

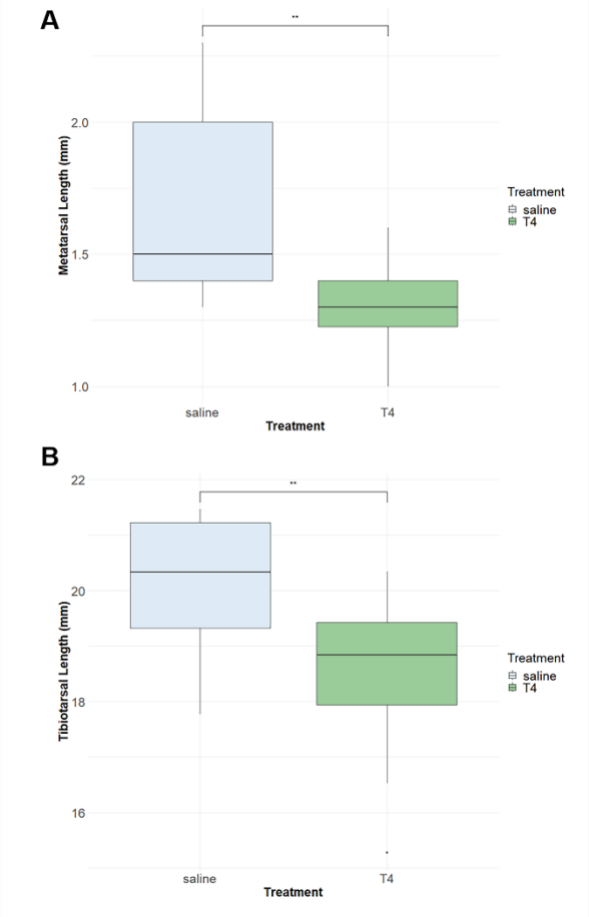

Figure 5. Box plot summarizing the distribution of measured (A) tibiotarsal length and (B) metatarsal length from chicken embryos on 19 dpf following thyroxine exposure. (A) The third metatarsal length was 16.4 ± 0.32 mm following saline exposure (N=16) but decreased to 13.1 ± 0.16 mm (N=14) following thyroxine exposure ($p<0.01$). (B) The tibiotarsal length was 20.1 ± 1.18 mm following saline exposure (N=15) but decreased to 18.6 ± 1.42 mm (N=14) following thyroxine exposure ($p<0.01$).. The box encompasses the interquartile range of concentrations with a line marking the median and "whiskers" extending from the box to the minimum and maximum values; comparisons were made between measured metatarsal and tibiotarsal lengths of those embryos receiving the control treatment (saline) and each T_4 treatment; significance was determined using the Welch's two student t-test (**, $p<0.01$).

Since T_3 is the more potent TH, we hypothesized that the injected T_3 was not being metabolized quickly enough to regulate TH bioactivity and leading to the death of the exposed embryos (Peeters & Visser, 2000). Therefore, this result led the decision to eliminate T_3 from injection solutions and following experiments used a dose of 1.0 µg T_4 and 0.5 µg T_4. These injections still resulted in a low survivability rate of 20% and 27%, respectively. While an improvement, a survivability rate of 27% is not optimal for creating a model of study intended to be used to evaluate thyrotoxicosis during development, which even in the most life-threatening conditions only has a mortality rate of 8%-25% (Lee *et al*., 2023).

To improve survivability further, the sequential experiment tested graded doses of T_4 and evaluated both viability and body mass. The fertilized chicken embryos were exposed to saline and T_4 at 25 pg, 0.25 ng, 2.5 ng, 25 ng, 0.25 µg on both 11 dpf and 15 dpf. These days of exposure were chosen based on our previous survivability observations. Of the embryos that did not survive the T_4 exposure, it was the second day post injection when they were reported nonviable. Therefore, we planned for three days between injections in anticipation of improving our survivability rates by 19 dpf. Body mass at 19 dpf was recorded to determine if increasing T_4 exposure correlated to weight loss in this study. We expected that if we were effectively inducing thyrotoxicosis in these chicken embryos, that body mass would be significantly less than that of the saline controls since maternal hyperthyroidism correlates with a low birth weight (Derakhshan *et al.*, 2020). Overall, we were able to determine that the 25 ng T_4 exposure on 11 dpf and 15 dpf resulted in an acceptable survivability of over 90% with a 20% reduction in body mass ($p<0.001$). To validate that this experimental design is an effective model of induced thyrotoxicosis, we continued to use this dosage regimen in further studies evaluating additional morphological and molecular alterations to the exposed chicken embryos.

In the developing chicken embryo, endogenous levels of T_4 steadily rise towards the end of development, particularly after 11 dpf (Liu & Porter, 2004). In our model, we rely on diffusion for the exogenous T_4 at 11 dpf and 15 dpf to enter circulation. Thyroxine is a small lipophilic molecule composed of two linked tyrosine amino acids with four iodine atoms attached at the 3-, 3', 5- and 5'-positions on the aromatic rings. After being introduced into the air cell of the fertilized chicken egg, T_4 must diffuse through the outer membrane known as the chorioallantosis, and the inner membrane called the chorioendothelium, before entering into the capillary blood (Tazawa, 1980). Therefore, we wanted to ensure that the molecular and morphological effects that we observed in our study was because of our induced thyrotoxicosis with the uptake of our treatment into the embryonic circulation. We expected to find increased levels of T_4 with an accompanied decrease in thyroid-stimulating hormone (TSH) in circulation because with rising free T_4 (FT_4) levels, there is a negative feedback mechanism causing a decrease in TSH from the pituitary. On 19 dpf, we observed a 100% increase in circulating levels of T_4 ($p<0.01$). Additionally, while levels of circulating TSH did decline by 21%, this decrease was statistically insignificant ($p=0.3885$). Our ELISA measured levels of both bound and free forms of T_4. However, only FT_4 levels inhibit TSH release from the pituitary. We thus theorize that the insignificant decrease in TSH compared to the predicted increase in T_4 may be a result of the quantified levels of T_4 including a large percentage of bound forms; and therefore, our model would not demonstrate a large negative feedback effect on TSH (Shahid *et al.*, 2023). This result could be confirmed with a more in-depth analysis that would determine the specific fraction of unbound FT_4 in circulation.

Thyroid hormones enter target cells via specific membrane transporters before T_4 is converted into the active form, T_3 (Leitch *et al*., 2020). In the liver, a major site for the storage and metabolism of thyroid hormones, iodothyronine deiodinase type 1 (DIO1) is the most abundant deiodinase that activates T_4 to T_3 by removing the 5'-iodine atom (Sheridan, 1983; Leitch *et al*., 2020; Peeters & Visser, 2000; Bruinstroop *et al*., 2023). Iodothyronine deiodinase type 3 (DIO3), on the contrary, deactivates both T_4 and T_3 by removing the 5-iodine atom converting them to reverse T_3 (rT_3) or T_2, respectively (Leitch et al., 2020). Expression patterns between DIO1 and DIO3 may differ between stages of development so the intracellular activity of T_3 resulting from its activation or inactivation is locally regulated by the relative expression of these deiodinases. While DIO1 is known to be abundant in the liver, DIO3 is highly

expressed during development as a protective mechanism for tissues against higher levels of intracellular T_3 and T_4. In the livers collected from 19 dpf chicken embryos following thyroxine exposure, we observed a decrease in *DIO3* mRNA expression ($p<0.01$) while *DIO1* mRNA expression was unchanged ($p=0.658$). This decrease in expression of DIO3 indicates a decrease in DIO3 activity compared with normal DIO1 activity allowing T_4 to be converted to T_3 promoting metabolism in the liver and overwhelming the protective effects of DIO3 inactivation of excess THs. In our model, we additionally observed a decrease in liver mass following thyroxine exposure (data not shown, $p<0.01$) which could also be indicative of increased metabolism of hepatocytes within the liver. The specific pathway that regulates the altered expression levels of DIO3 is unknown; however, the liver is capable of metabolizing thyroid hormones through other mechanisms, including sulfation by sulfotransferases. Therefore, DIO3 expression levels may be modified through one of these alternative methods (Bruinstroop *et al*., 2023). Despite the gaps in understanding the mechanism for these observed results, they do support the susceptibility of the liver in our model to increased circulating T_4 levels.

After confirming that we had successfully induced thyrotoxicosis, we chose to examine body, metatarsal, and tibiotarsal length to represent quantifiable means of assessing interstitial growth following T_4 exposure as bone is a common target organ of THs. Additionally, the leg bones are reported to be more severely affected by excess hormones than wing bones (Lawson, 1961). We observed attenuated growth in overall body length ($p<0.001$), third metatarsal length ($p<0.01$), and tibiotarsal length ($p<0.01$) in our study. Similarly, Lawson (1961), who reported that there is a differential growth response to T_4, also observed attenuated growth of the tibiotarsus and third metatarsus following treatment with triiodothyronine *in vitro* (Lawson, 1961). While generally, thyroid hormones have been known to augment interstitial bone growth during postnatal development, it has been shown that congenital hyperthyroidism results in advanced bone aging in the developing skeleton (Williams, 2013). Therefore, our findings are in consonance with our model of induced thyrotoxicosis during embryonic development.

Conclusion

While the chicken embryo has long been a reputable organism for various molecular and morphological studies in developmental biology, we now have established it to be a particularly useful vertebrate model for studying the effects of induced-thyrotoxicosis during embryonic development. The established dose regimen is effective at altering TH regulation and impacting physiology, including altered skeletal growth. Although other organ systems are yet to be studied using this model, we conclude that this avian model of induced-thyrotoxicosis will be valuable for studying the molecular and morphological effects of thyrotoxicosis-induced craniofacial abnormalities, including craniosynostosis.

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