

TESTOSTERONE (T) AND TRIIODOTHYRONINE (T3) IN FRANKLIN'S GULL
(*LEUCOPHAEUS PIPIXCAN*) EGGS

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Title

Testosterone (T) and triiodothyronine (T3) in Franklin's Gull (*Leucophaeus
pipixcan*) eggs.

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ABSTRACT

Maternally-derived hormones are known to influence the growth and development of offspring. The differential deposition of these maternally-derived hormones into egg yolk is one way by which females can alter and impact their chicks' survival. Yolk constituents, especially testosterone, have been described for a wide variety of species. However, few studies have focused on multiple maternally-derived hormones regulated by independent axis in the endocrine system, these of which have mainly focused on corticosterone and testosterone. We determined within and among female variation in testosterone and triiodothyronine concentrations in egg yolks of two free-living Franklin's gull (*Leucophaeus pipixcan*) populations. We found that testosterone, not triiodothyronine, concentrations increased within the clutch with the third laid egg having significantly higher concentrations than the first laid egg. We also found that both testosterone and triiodothyronine concentrations increase within the breeding season with late season clutches having significantly higher hormone concentrations than early season clutches.

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TESTOSTERONE AND TRIIODOTHYRONINE IN FRANKLIN'S GULL EGGS¹

Introduction

Changes in resource allocation to eggs are one way female birds can alter and influence their young during development in the egg, and after hatching. For example, maternal androgens can increase the rate of growth and development of embryos as well as hatchlings (Eising et al. 2001). Variation in maternally-derived constituents of eggs is manifested both within and among females. If egg constituents affect offspring performance, this implies that offspring survival may differ relative to same-clutch siblings, as well as unrelated individuals in the same cohort.

The patterns of within-female variation of maternally-derived hormones in avian yolks have been studied in a variety of species; however, no consistent pattern is evident. Most of the research on, and understanding of, maternally-derived hormones in egg yolks is based on patterns of yolk testosterone. Testosterone concentrations increase across the laying sequence in Canary (*Serinus canaria*) (Schwabl 1993; Schwabl 1996), Black Headed Gull (*Larus ridibundus*) (Groothuis and Schwabl 2002; Müller et al. 2004), European Starling (*Sturnus vulgaris*) (Pilz et al. 2003), Yellow-legged Gulls (*Larus michahellis*) (Rubolini et al. 2011), and Black Tailed Gulls (*Larus crassirostris*) (Tomita et al. 2011). Testosterone concentrations decrease across the laying sequence in Zebra Finch (*Poephila guttata*) (Schwabl 1993; Gil et al. 1999), Cattle Egret (*Bubulcus ibis*) (Schwabl et al. 1997), Pied Flycatcher (*Ficedula hypoleuca*) (Tobler et al. 2007), Spotless Starling (*Sturnus unicolor*) (Lopez-Rull et al. 2010), and Screech Owl (*Megascops asio*) (Hahn 2011). Concentrations increase then decrease across the sequence in American Coot (*Fulica americana*) (Reed and Vleck 2001) and Canada Goose (*Branta canadensis maxima*)

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(Boonstra et al. 2009). There is no trend in Tree Swallow (*Tachycineta bicolor*) (Wittingham and Schwabl 2002). There are two hypotheses to explain the adaptive consequences of these changes in testosterone across the laying sequence: 1) the hatching asynchrony adjustment hypothesis (Groothuis et al. 2005b), and 2) the brood reduction hypothesis (Lack 1947). The hatching asynchrony adjustment hypothesis proposes that females increase testosterone levels in the last-laid egg to mitigate competitive disadvantages faced by later-hatching chicks due to hatching asynchrony (Groothuis et al. 2005b), while the brood reduction hypothesis proposes that, under stressful environmental conditions, some species decrease the testosterone level across the laying sequence, which increases the likelihood of siblicide against chicks from later-laid eggs (Schwabl et al. 1997).

Testosterone is an anabolic steroid hormone that is known to increase growth rates and development of hatchlings. Higher concentrations of testosterone in the later-laid eggs of a clutch are a potential mechanism by which a female can accelerate growth and development of the corresponding later-hatching chicks to mitigate the effects of hatching asynchrony. Higher levels of testosterone have been shown to increase begging behavior, feeding bouts, pecking rates and growth of the hatching muscle (*musculus complexus*), resulting in larger chicks (Eising and Groothuis 2003; Noguera et al. 2013; Lipar and Ketterson 2000). Higher levels of testosterone can also have negative consequences for offspring. For example, Black-Headed gull eggs with experimentally increased testosterone concentrations produced chicks with decreased immune system function at hatching (Groothuis et al. 2005a). This suggests that there may be a trade-off between enhanced growth versus diminished immune function associated with testosterone concentration in the yolk.

Females may also pay costs of egg yolk hormones, which can play a role in determining among-female differences in yolk hormones. Female Canaries on a low quality diet tend to have less testosterone in their eggs compared to females on a high quality diet, suggesting costs for females to deposit testosterone into the yolk of her eggs (Vergauwen et al. 2012). In some species, females nesting in higher densities have higher levels of yolk hormones in their eggs (Schwabl 1997; Reed and Vleck 2001). Furthermore, yolk testosterone levels can be heritable from mother to daughter (Ruuskanen et al. 2016b), suggesting that both current environmental conditions and genetic factors affect maternal hormone deposition, and the quality of the chicks produced from the eggs.

While our understanding of maternally-derived hormones has largely developed from studies of androgens such as testosterone, recent studies have focused on effects of thyroid hormones. Two thyroid hormones, triiodothyronine (T3) and thyroxine (T4), vary with photoperiod and regulate migratory behavior in birds (Pant and Chandola-Saklani 1993; Wingfield et al. 1996; Perez et al. 2016). Because these hormones vary seasonally, and influence migratory behavior, the levels of these hormones may differ in eggs laid during different periods of the breeding season. When full clutches of Great Tits (*Parus major*) were experimentally manipulated using a thyroid injection into the egg yolk, prior to the initiation of incubation, the thyroid treatment increased growth rates in males but decreased growth rates in females, and there were no treatment effects on motor coordination, stress handling, or metabolic rates of the nestlings (Ruuskanen et al. 2016a). Hence, maternally-derived thyroid hormones in eggs may have similar growth effects as maternally-derived androgens, but are produced through a different hormone axis (hypothalamus-pituitary-thyroid axis as opposed to the hypothalamus-pituitary-gonad axis) in the mother.

Seasonal differences in the timing of egg production impact offspring fitness. Within-season differences in chick growth, development and survival have been ascribed to changing environments (e.g., food availability, temperature, predator exposure) (reviewed in Sockman et al. 2006). However, Franklin's gull (*Leucophaeus pipixcan*) chicks from early and late season eggs reared in a common garden experiment, exhibited differential growth and development depending on when during the season the egg was produced (with chicks from late-season eggs gained mass and grew wing feathers faster than chicks from early season eggs) (Reed and Clark 2016). These results suggest that seasonal differences in offspring performance can be programmed at the egg stage, either through maternal investments in egg components or possibly genetic differences associated with season.

To explore how maternally derived hormones may contribute to seasonal differences in offspring growth and development in Franklin's gulls we describe patterns of within and among female variation in maternally-derived yolk testosterone and triiodothyronine levels in their eggs. We explore this variation as a consequence of variation in laying date and laying sequence for two free-living gull populations.

Methods

Egg Collection

We collected freshly-laid eggs in 2015 and 2016 from three Franklin's gull colonies located in North Dakota to characterize variation within and among clutches. In 2015 we located nests with a single egg, used a floatation method (Nol and Blokpoel 1983; Ackerman and Eagles-Smith 2010) to determine whether the egg was laid that day and if it was, we removed the egg from the nest and replaced it with a marked egg from another female. We returned to the nest after 24 hours to check for an additional egg, which was similarly removed and replaced with

another marked egg from a different female. We continued visiting the nest daily until the female stopped producing eggs. Following this protocol, we collected 24 eggs from three-egg clutches of eight females nesting at a large wetland (48°09'N, 98°25'W) in Nelson County, North Dakota from May 19, 2015 to May 22, 2015 (when nest initiation in the colony was at the peak). In 2016 we collected freshly-laid (verified by flotation), first eggs from nests at the beginning of the nest initiation period (i.e. early season, within the first week of nest initiation in the colony) and nearing the end of the nest initiation period (i.e. late season, approximately in the last week of nest initiation in the colony) at two nesting colonies in North Dakota. We collected 12 eggs on May 20, 2016 and 15 eggs on May 27, 2016 at a colony located on J. Clark Salyer National Wildlife Refuge (NWR) (48°61'N, 100°71'W) in McHenry County, North Dakota. We collected 10 eggs on May 21, 2016 and 11 eggs on May 28, 2016 at a colony located on a large wetland (48°98'N, 102°68'W) in Burke County, North Dakota. We measured mass (± 0.01 g), length (± 0.1 mm) and breadth (± 0.1 mm) of all eggs within 12 hours of collection, separated the yolk from the albumen using a separation spoon, and stored the yolk at -20 C until hormone analyses were conducted.

Testosterone Analysis

We determined testosterone concentrations in yolk using enzyme-linked immunosorbent assay (ELISA). Yolk samples that were serially diluted resulted in a displacement curve that was parallel to the given testosterone standard. Testosterone (T) was extracted from the yolk following the protocol used by Schwabl (1993), but modified for use with Franklin's gull yolk and an ELISA. In brief, we mixed approximately 17 mg of yolk with 1 ml of double distilled water, and placed the sample in the fridge overnight. On the second day of extraction, the sample was extracted three times in 4.0 ml of petroleum ether and diethyl ether (30:70, vol/vol),

the sample was snap frozen, and the ether phase was decanted and dried down under a stream of N₂. The precipitate was suspended in 1.0 ml of 90% ethanol and stored overnight at -4° C. On the third day of extraction we added 2.0 ml of hexane to the ethanol phase and collected the lower phase (this step was repeated twice more for a total of three washes) then centrifuged at 900 rcf for 5 minutes, decanted into a new tube, and dried down under N₂. We suspended the precipitate in 550 µl of assay buffer supplied with the ELISA kit (Enzo Life Sciences, Farmingdale, NY), then stored the suspension at 4° C overnight. Testosterone levels in the suspension were then measured following the ELISA manufacturer's protocol (Enzo Life Sciences, Farmingdale, NY). We converted T levels to concentrations per mg wet yolk based on the amount of yolk used in the extraction.

Triiodothyronine Analysis

We determined triiodothyronine concentrations in yolk using ELISA. Yolk samples that were serially diluted resulted in a displacement curve that was parallel to the given triiodothyronine standards. Triiodothyronine (T₃) was extracted from the yolk following the protocol used by Ho and colleagues (Ho et al. 2011) with modifications for use with Franklin's gull eggs and an ELISA. Briefly, we placed approximately 500 mg of yolk into a 15 ml glass conical tube, added 2.0 ml of methanol, centrifuged at 1200 rcf for 10 min, and decanted the supernatant. The yolk was suspended in 1.0 ml of methanol, centrifuged at 1200 rcf for 10 min, and decanted into a separate tube. We added 5.0 ml of chloroform and 0.5 ml of 2 mol ammonium hydroxide to each tube, collected the upper phase (combining both upper phases, from the same samples, into the same tube) and dried the sample in a vacuum concentrator (Savant model SVC100H). We suspended the precipitate in 1.0 ml of 2 mol ammonium hydroxide, centrifuged at 1200 rcf for 10 min, and decanted into a new tube to which we added

1.0 ml of chloroform, then centrifuged at 1200 rcf for 10 min, collected the upper phase and dried the sample in a vacuum concentrator. We suspended the precipitate in 170 μ l of phosphate buffer solution and stored the sample at 4° C overnight. Triiodothyronine levels in the suspended precipitate were then measured using an ELISA kit (Monobind Inc, Lake Forest, CA) in which we modified the manufacturer's protocol by lowering the concentration of Working Reagent A T3 enzyme conjugate solution from 100 μ l per well to 75 μ l per well and lengthening the incubation period from 1 hour to 2 hours. We converted T3 levels to concentrations per mg wet yolk based on the amount of yolk used in the extraction.

Statistical Analysis

We analyzed within-clutch and among-clutch variation in T and T3 concentrations in yolk using general linear models. Concentrations of T and T3 followed a log-Normal distribution, so we log-transformed concentrations for statistical analysis. For within-clutch variation, we modeled (log-transformed) T (and T3) concentrations using a model with a fixed effect of position (as a categorical variable) in the laying sequence and a random effect for female, a model with a fixed effect of days since the start of laying and a random effect for female. Within-clutch differences in T and T3 concentrations among positions in the laying sequence were compared by a post-hoc Tukey's HSD test. For among-clutch variation, we modeled T (and T3) concentration using a model with a fixed effect of season (i.e., early- versus late-season), location and the interaction between season and location. However, effects of location and the interaction of season and location were not significant, so we present results from a reduced model in which only the effect of season is included. We also examined correlation between T and T3 concentrations in the among-clutch analysis using the Pearson

correlation coefficient. We assumed statistical significance at $\alpha=0.05$. All statistical analyses were conducted using JMP 13 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

We extracted and successfully quantified T and T3 concentrations in the yolks from 72 eggs. In the 2015 collection, we successfully determined T and T3 concentrations in all 24 eggs (from eight clutches containing three eggs each) for analyses of within-clutch variation. The coefficient of variation between sample duplicates was $< 12\%$ for all the samples. In the 2016 collection, we successfully determined T concentrations in 48 of the first-laid eggs (12 from J. Clark Salyer NWR on May 20, 10 from the Burke County site on May 21, 15 from J. Clark Salyer NWR on May 27, and 11 from the Burke County site on May 28). However, we only obtained T3 concentrations for 46 of these eggs because the coefficient of variation between duplicates in one of the May 28 samples from Burke County exceeded 15% and we were unable to perform the T3 extraction on one of the May 27 samples from J. Clark Salyer. The coefficients of variation between duplicates for all other samples were $< 13\%$. Further, Inter-assay variation in T and T3 levels were 5.1 and 5.9 respectively. Intra-assay variation in T and T3 ranged from 3.7 to 3.3 and 8.3 to 6.1 respectively.

Concentrations of T in yolk increased with position in the laying sequence, but concentrations of T3 did not differ with position. Position in the laying sequence and female explained over 45% of the variation in log-transformed T concentrations ($F_{2,14} = 4.61$, $P = 0.029$, $r^2 = 0.47$), with less than 17% of the variance due to the female random effect. The Tukey HSD test indicated log concentration of T in the first egg of the clutch (least-square mean \pm SE of 1.97 ± 0.26) was not different from log concentration of the second egg (2.36 ± 0.26), but was significantly less than log concentration of T in the third-laid egg (2.98 ± 0.26) (Figure 1A).

Position in the laying sequence and female did not explain a significant amount of variation in log-transformed T3 concentrations ($F_{2,14} = 3.23$, $P = 0.070$, $r^2 = 0.45$), with less than 19% of the variation due to the female random effect (Figure 1B). Testosterone levels increase in the egg from the start of laying ($F_{1,22} = 8.43$, $P = 0.008$, $r^2 = 0.27$; Figure 2) and also increase significantly since the previous egg was laid ($F_{1,22} = 6.84$, $P = 0.016$, $r^2 = 0.24$).

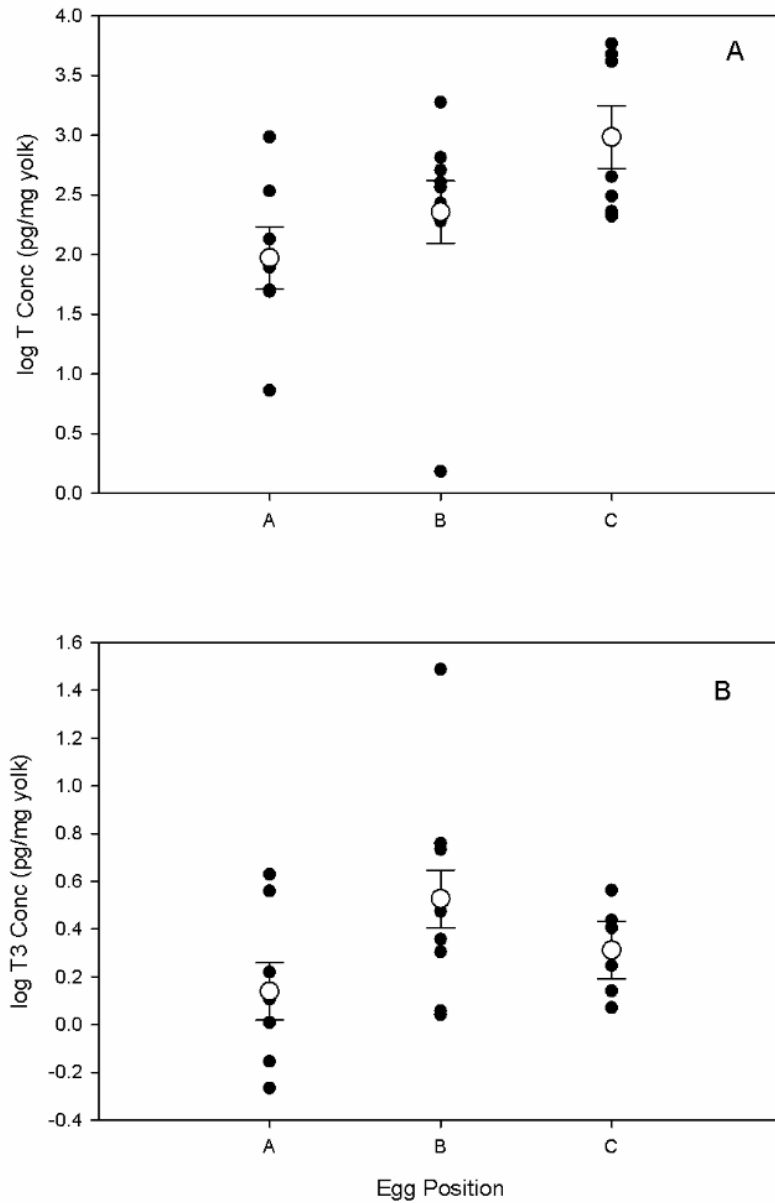


Figure 1. Within clutch concentrations of A) testosterone and B) triiodothyronine (filled circles represent observed values, open circles represent means, and bars are SE).

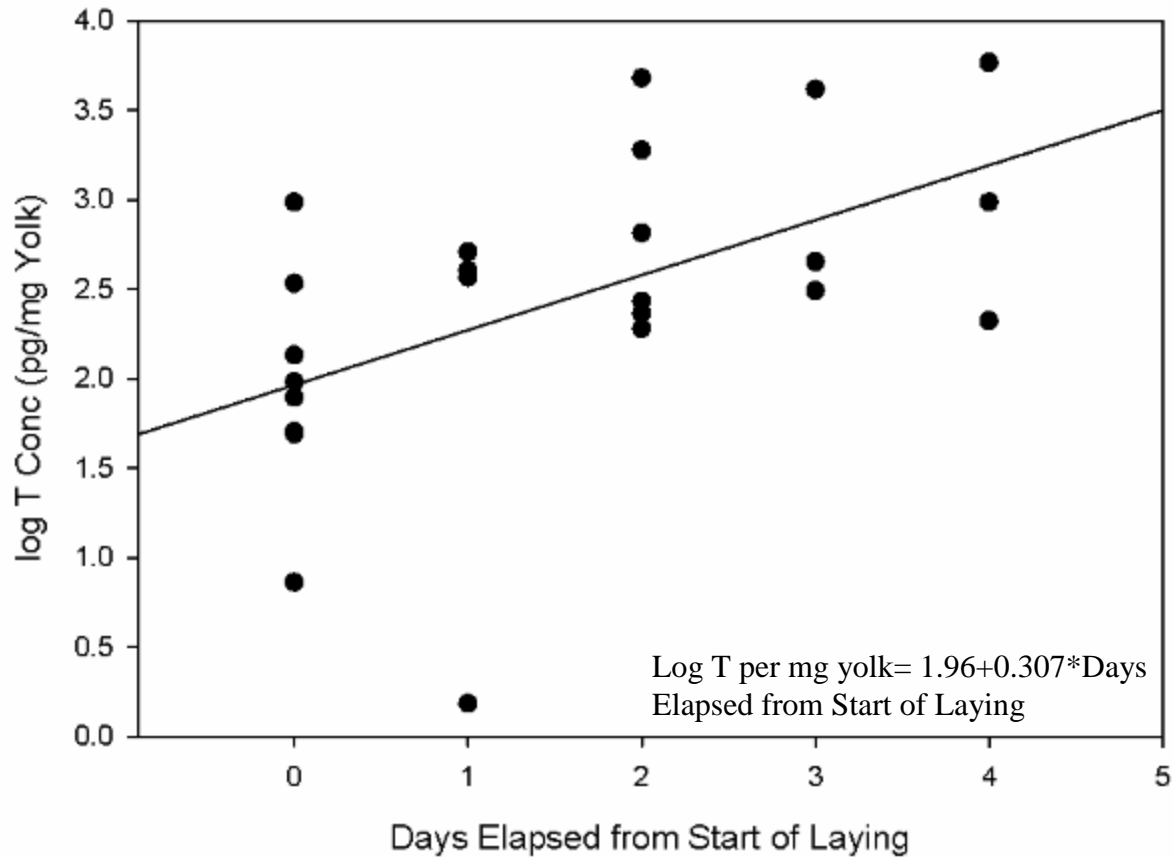


Figure 2. Within clutch concentrations of testosterone by days elapsed from the start of laying (filled circles represent observed values and line represents line of best fit).

Maternally derived yolk T and T3 concentrations increased across the laying season.

Log-transformed concentrations of T ranged from 0.84 to 3.61 pg/mg yolk, with concentrations from eggs laid early (mean \pm standard error of 1.95 ± 0.12 pg/mg yolk) significantly lower than concentrations from eggs laid late in the season (2.37 ± 0.11 pg/mg yolk) ($F = 6.46$, $P = 0.0145$, $r^2 = 0.12$; Figure 3A). Log-transformed concentrations of T3 ranged from -0.50 to 0.37 pg/mg yolk with concentrations from eggs laid early (mean \pm standard error of -0.166 ± 0.04 pg/mg yolk) significantly lower than concentrations from eggs laid late in the season (-0.029 ± 0.04 pg/mg yolk) ($F = 5.19$, $P = 0.028$, $r^2 = 0.11$; Figure 3B). Although concentrations of both T and T3 increased significantly across the season, there was no significant correlation between concentrations of T and T3 ($F_{1,44} = 0.505$, $P = 0.481$, $r^2 = 0.011$).

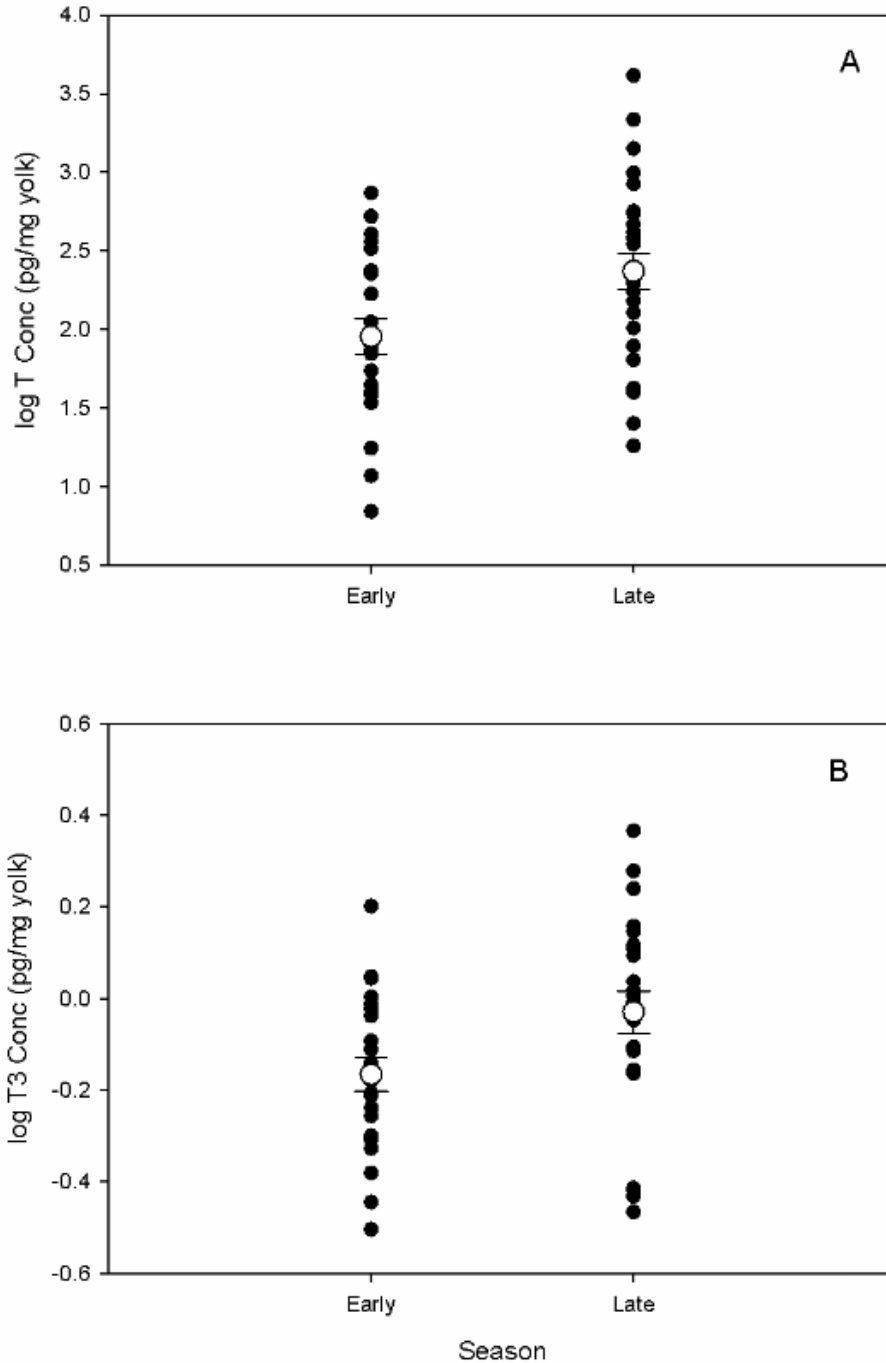


Figure 3. Among clutch concentrations of A) testosterone and B) triiodothyronine (filled circles represent observed values, open circles represent means, and bars are SE).

Discussion

In Franklin's gull eggs, we found different patterns of within-clutch variation in T and T3, but similar patterns of within-season variation in maternally-derived T and T3 hormone

levels. These hormones are regulated by separate hormone axes, yet both hormones affect growth and development. Yolk T levels increase across the laying sequence in Franklin's gull, but yolk T3 levels showed no pattern across the laying sequence. Moreover, T concentrations in yolk increased from the start of laying as well as from the time the previous egg was laid. Both T and T3 concentrations in egg yolks increased as the breeding season progressed, but the concentrations were not correlated with each other. That is, females depositing high concentrations of T in an egg do not necessarily deposit high concentrations of T3 in the egg. These results suggest that both T and T3 may act as independent mechanisms, regulated along separate hormone axes in the mother, to facilitate increased growth and development rates of Franklin's gull chicks produced later in the breeding season (Reed and Clark 2016).

The pattern of increasing T concentrations across the laying sequence supports the hatching asynchrony adjustment hypothesis in Franklin's gull. Franklin's gull chicks hatch over a period of 2-3 days, with the last chick experiencing greater competition for food and nest space than the older siblings (Burger and Gochfeld 2009). In other gull species, higher levels of yolk T increase chick aggression, begging behaviors, and feeding rates (Eising and Groothuis 2003). Increased aggression may be especially critical for survival of the youngest chick when competing with their older, larger siblings.

Yolk T concentrations were not only highest in the last-laid eggs, but also increased as the days elapsed between egg laying increased. For example, when 48 hours elapsed between eggs one and two for a female, T concentrations present in her second egg were relatively higher than concentrations in the second egg of a female for which 24 hours elapsed between eggs one and two. The mechanism by which yolk T is deposited in eggs is unclear, although passive diffusion from the gonadal tissues and blood vessels surrounding the developing yolk is one of

the most parsimonious explanations (reviewed in Groothuis and Schwabl 2008). The pattern of yolk T in gull eggs suggests that yolks of females who skipped a day may have been exposed to T in the gonad for a longer period of time, allowing more time for diffusion of T from the blood to the yolk to reach equilibrium, or that by skipping a day these females have higher concentrations of T in circulation driving the diffusion of T into yolks. It is thought that the surge of luteinizing hormone (LH) associated with ovulation and T secretion creates a positive feedback loop and may ultimately determine the amount of T that diffuses into the yolk (Okuliarova et al. 2017). While plasma T concentrations are changing on both longer (seasonal) and shorter (ovulation) time scales, plasma T3 concentrations have been shown to increase a month before the beginning of laying and are higher in early breeding birds than in late breeding birds during this time, suggesting that T3 concentrations in egg yolks may only change across longer time scales (Chastel et al. 2003).

The patterns of T and T3 in egg yolks are likely reflection the seasonal and short term dynamics of the different hormone axes represented. The hypothalamus-pituitary-gonad axis initiates activity through gonadotropin releasing hormones (GnRH) from the hypothalamus stimulating follicle stimulating hormone (FSH) and LH from the pituitary to affect hormone secretion from the gonad and resulting changes in behaviors and physiology associated with reproduction, in addition to short-term hormone changes associated with ovulation. The hypothalamus-pituitary-thyroid axis acts through thyrotropin releasing hormone (TRH) from the hypothalamus stimulating thyroid stimulating hormone (TSH) from the pituitary and T3 from the thyroid gland. This axis is also sensitive to seasonal changes associated with molting and migration (Perez et al. 2018), however there is little evidence that the T3 axis exhibits shorter term changes associated with ovulation or egg production. Thyroid hormone secretion has been

associated with differences in early and late nesting house sparrows (Chastel et al. 2003). In house sparrows, pre-breeding plasma T3 levels were higher in birds that ended up breeding early in the season than in birds that ended up breeding late in the season (Chastel et al. 2003). These data suggest that there could be differences at the time of egg production. The T axis seems to be working on both a short-term time scale associated with ovulation as well as a long-term scale associated with the length of the breeding season, whereas the T3 axis only seems to change on the longer time scale of season. In our study, all eggs were collected prior to the summer solstice, during the period of increasing day length, but may reflect changes in adult physiology as birds transition from breeding to migration.

The longer-term changes in T and T3 drive seasonal transitions of adult behavior and physiology with consequences for offspring development. Although less studied than T, T3 appears to have similar positive effects for growth and development. Triiodothyronine is important for molt regulation and migration processes and has also been shown to decrease incubation time in turtles (McGlashan et al. 2017). Triiodothyronine is also critical for embryonic growth and differentiation of several tissues associated with the muscular, skeletal, and nervous system (Yamaguchi et al. 2017).

Higher levels of T3 in late season eggs may be one mechanism by which these embryos program faster growth and development. Later hatching chicks have less time to mature and grow before having to initiate migration. Therefore, shorter incubation periods combined with higher T3 and T levels may help these chicks mitigate the effects of hatching late in the season. The seasonal increase in both T3 and T in Franklin's gull egg yolks and the general growth enhancing effects of these hormones are consistent with patterns of increased growth rate and development in chicks hatching from eggs laid later in the season (Clark and Reed 2012; Reed

and Clark 2016). For example, Franklin's gull chicks hatched later in the season have shorter incubation times (less by ~ 24 hours) (Clark and Reed 2012). This is consistent with higher T3 levels we observed in later-laid eggs. When gull chicks from early and late-season eggs were raised under common garden conditions, late season chicks exhibited faster growth in both body mass and wing area (Reed and Clark 2016). Given the influence of T3 on molt in adults, increased T3 levels in late season eggs could play a role in this faster rate of development.

There have been a limited number of studies focusing on multiple maternally-derived hormones regulated by independent axes in the endocrine system and these have largely focused on corticosterone and testosterone. There is also very little information in the field on triiodothyronine with regards to seasonal trends of concentration in eggs and the impact T3 plays on the growth and development of embryos and chicks. We observed similar seasonal patterns of testosterone and triiodothyronine, even though concentrations of the two hormones were not correlated. We also observed an increase of testosterone within the clutch. These patterns are consistent with selection for fast offspring growth later in the season for species with a distinct breeding period. Within and among clutch differences in hormones needs to continue to be studied and should expand to look at new hormones with possible links to growth and development in both embryos and chicks.

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