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Testosterone and Triiodothyronine 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 their chicks' growth or 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 axes in the endocrine system, and those studies that have, mainly focus on corticosterone and testosterone. In this study, within and among female variation in testosterone and triiodothyronine concentrations in egg yolks were measured in three free-living Franklin's Gull (*Leucophaeus pipixcan*) populations. Testosterone (T), but not triiodothyronine (T3), concentrations increase within a female's clutch such that eggs laid later in the clutch have increasingly higher concentrations (from a mean of 1.97 log pg T/mg yolk for the first egg to 2.98 log pg T/mg yolk for the third egg compared to a mean of 0.14 log pg T3/mg yolk for the first egg to 0.31 log pg T3/mg yolk for the third egg). Both testosterone and triiodothyronine concentrations increase among females across the breeding season such that eggs laid later in the season have significantly higher hormone concentrations (mean of 2.37 log pg T/mg yolk and mean of -0.03 log pg T3/mg yolk) than eggs laid early in the season (mean of 1.95 log pg T/mg yolk and mean of -0.17 log pg T3/mg yolk). Received 21 January 2019, accepted 25 June 2019.

Key words.—eggs, Franklin's Gull, hormones, *Leucophaeus pipixcan*, maternal effects, season, testosterone, triiodothyronine.

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Female birds can alter and influence their young during development in the egg and after hatching through allocation of resources to eggs. 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, 1996), European Starling (*Sturnus vulgaris*) (Pilz *et al.* 2003), Black-headed Gull (*Larus ridibundus*) (Müller *et al.* 2004; Groothuis and Schwabl 2008), Yellow-legged Gull (*Larus michahellis*) (Rubolini *et al.* 2011), and Black-tailed Gull (*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 1997), Pied Flycatcher (*Ficedula hypoleuca*) (Tobler *et al.* 2007), Spotless Starling (*Sturnus unicolor*) (López-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 Giant Canada Goose (*Branta canadensis maxima*) (Boonstra *et al.* 2009). There is no trend in testosterone levels within Tree Swallow (*Tachycineta bicolor*) clutches (Whittingham and Schwabl 2002). Two hypotheses have been proposed 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 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 (Lipar and Ketterson 2000; Eising and Groothuis 2003; Noguera *et al.* 2013). Higher levels of testosterone can also have negative consequences for offspring. For example, Black-headed Gull eggs with experimentally elevated 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.

Among-female differences in yolk hormones may reflect differences in female

quality or costs of hormone levels to adult females. Captive female canaries fed a low-quality diet tend to have less testosterone in their eggs compared to females fed a high-quality diet, suggesting physiological 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, variation in 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 Chandolasaklani 1993; Wingfield *et al.* 1996; Pérez *et al.* 2016). Because these hormones vary seasonally in adults, the levels of these hormones may differ in eggs laid during different periods of the breeding season. When yolk thyroid concentrations in the eggs of full clutches of Great Tits (*Parus major*) were experimentally elevated 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, devel-

opment 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 gaining mass and growing wing feathers faster than chicks from early season eggs; (Reed and Clark 2016). These results suggest that seasonal differences in offspring development can be programmed at the egg stage, either through maternal investments in egg components or genetic differences associated with season.

To explore how maternally derived hormones may contribute to seasonal differences in offspring growth and development in Franklin's Gull, 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 three free-living gull populations.

METHODS

Egg Collection

We collected freshly laid eggs in May 2015 and May 2016 from three Franklin's Gull colonies (Fig. 1) located in North Dakota, USA to characterize variation within and among females. 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. We assumed that all eggs collected from an individual nest were from the same female, and that eggs collected from different nests were from different females (Burger and Gochfeld 2009). Following this protocol, in 2015 we collected all 24 eggs from three-egg clutches of eight females nesting at a large wetland (48° 05' 23" N, 98° 12' 28" W; Fig. 1) in Nelson County, North Dakota, USA from 19 May 2015 to 22 May 2015 (when nest initiation in the colony was at the peak). In 2016, the

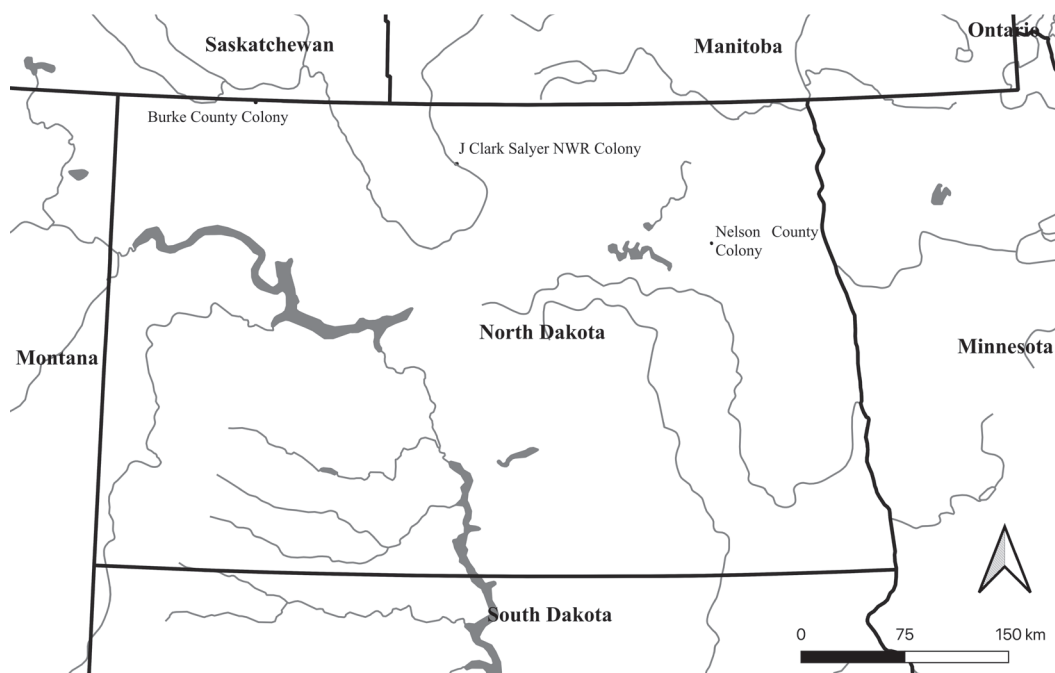


Figure 1. Location of Franklin's Gull (*Leucophaeus pipixcan*) colonies in North Dakota where eggs were collected for the study in 2015 (Nelson County Colony) and 2016 (J Clark Salyer NWR Colony and Burke County Colony).

Nelson County wetland was drained, and the Franklin's Gull colony relocated elsewhere. In 2016, we collected freshly-laid (verified by flotation), first-laid 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, USA. We collected 12 eggs on 20 May 2016 and 15 eggs on 27 May 2016 at a colony located on J. Clark Salyer National Wildlife Refuge (NWR; 48° 34' 29" N, 100° 38' 31" W; Fig. 1) in McHenry County, North Dakota, USA. We collected 10 eggs on 21 May 2016 and 11 eggs on 28 May 2016 at a colony located on a large wetland (48° 59' 3" N, 102° 41' 12" W; Fig. 1) in Burke County, North Dakota, USA. 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 1.0 ml of double distilled water and placed each sample in a refrigerator at 4°C overnight. On the second day of the extraction process, each sample was rinsed in 4.0 ml of petroleum ether and diethyl ether (30:70, vol/vol) three times (total of 12 ml ether mix), the sample was snap frozen, and the ether phase was decanted and dried down under a stream of nitrogen (N_2). 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 G for 5 minutes, decanted into a new tube, and dried under N_2 . We suspended the precipitate in 550 μl of assay buffer supplied with the ELISA kit (Enzo Life Sciences, Farmingdale, NY), and 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 fol-

lowing 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 μl 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 μl per well to 75 μl per well and lengthened the incubation period from 1 hour to 2 hours to optimize antibody binding in avian yolk. 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-female and among-female 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-female variation, we modeled log-transformed T and T3 concentrations using a model with a fixed effect of position in the laying sequence (as a categorical variable) and a random effect for female; and a model with a fixed effect of days since the start of laying and a random effect for female. Within-female differences in log-transformed T and T3 concentrations among positions in the laying sequence were compared by a post-hoc Tukey's honest significant difference (HSD) test. For among-female variation, we modeled T and T3 concentration using a model with a fixed effect of season (i.e., early- versus late-season), geographic 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 tested for pairwise correlation between T and T3 concentrations in the among-clutch analysis using the Pearson product moment (ρ) and a t-test. We assumed statistical significance at $\alpha = 0.05$. All statistical analyses were conducted using JMP Pro 14.0 statistical software (SAS Institute 2017).

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 collected on May 20, 10 from the Burke County site collected on May 21, 15 from J. Clark Salyer NWR collected on May 27, and 11 from the Burke County site collected on May 28). However, we only used 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%. Furthermore, inter-assay variation in T and T3 levels were 5.1 and 5.9 respectively. Intra-assay variation in T and T3 ranged from 3.3 to 3.7 and 6.1 to 8.3 respectively.

Within-female variation in yolk hormones indicates 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 = SE of 1.97 ± 0.26) was not different from log concentration of the second egg (2.36 ± 0.26 SE), but was significantly less than log concentration of T in the third laid egg (2.98 ± 0.26 SE) (Fig. 2A). 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

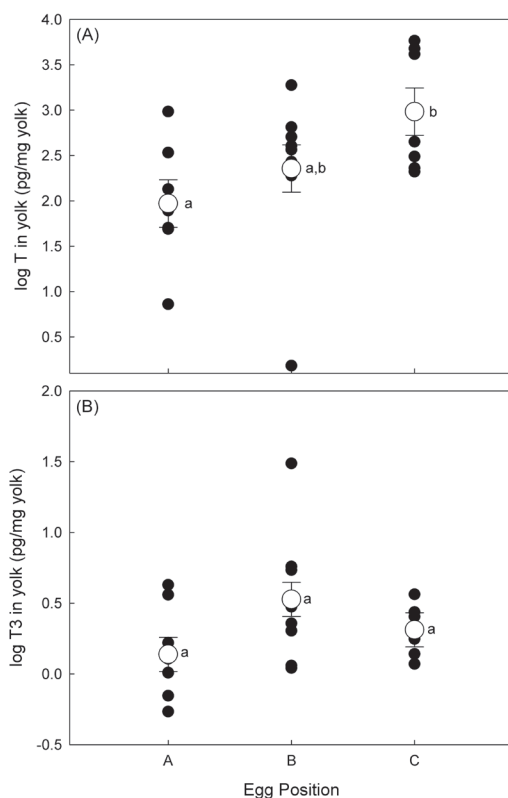


Figure 2. Within-female testosterone concentrations in yolk increase with position of the egg in the clutch (A), but triiodothyronine concentrations in yolk do not change with position (B) for Franklin's Gull (*Leucophaea pipixcan*) eggs (filled circles represent observed values, open circles represent least-square means with bars for SE, and small letters designate significantly different mean values).

random effect (Fig. 2B). Testosterone levels increased in the yolk from the start of laying ($F_{1,22} = 8.43$, $P = 0.008$, $r^2 = 0.27$; Fig. 3) and also increased with the number of days elapsed since the previous egg was laid ($F_{1,22} = 6.84$, $P = 0.016$, $r^2 = 0.24$).

Among-female variation in yolk hormones indicates 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 = 1.95 ± 0.12 SE pg/mg yolk) significantly lower than concentrations from eggs laid late in the season (2.37 ± 0.11 SE pg/mg yolk) ($F_{1,46} = 6.46$, $P = 0.0145$, $r^2 = 0.12$; Fig. 4A). Log-transformed concentra-

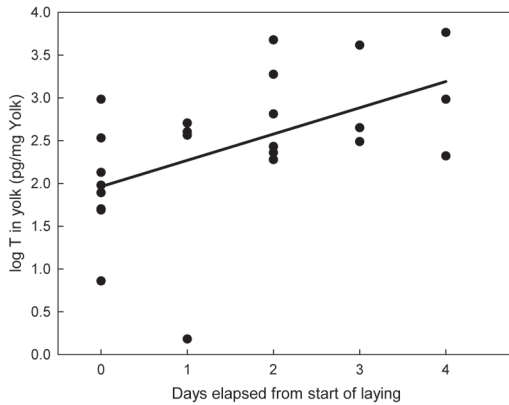


Figure 3. Within-female concentrations of testosterone in the yolk of Franklin's Gull (*Leucophaeus pipixcan*) eggs increase significantly with days elapsed from the start of laying (filled circles represent observed values and the solid line represents line of best fit).

tions of T3 ranged from -0.50 to 0.37 pg/mg yolk with concentrations from eggs laid early (mean = -0.17 ± 0.04 SE pg/mg yolk) significantly lower than concentrations from eggs laid late in the season (-0.03 ± 0.04 SE pg/mg yolk) ($F_{1,44} = 5.19$, $P = 0.028$, $r^2 = 0.11$; Fig. 4B). Although concentrations of both T and T3 increased significantly across the season, there was no significant correlation between concentrations of T and T3 ($\rho = 0.106$, $P = 0.481$, $n = 46$).

DISCUSSION

In Franklin's Gull eggs, we found different patterns of within-female variation in T and T3, but similar patterns of among-female (within-season) variation in maternally derived T and T3 hormone levels. Separate hormone axes regulate these hormones, 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

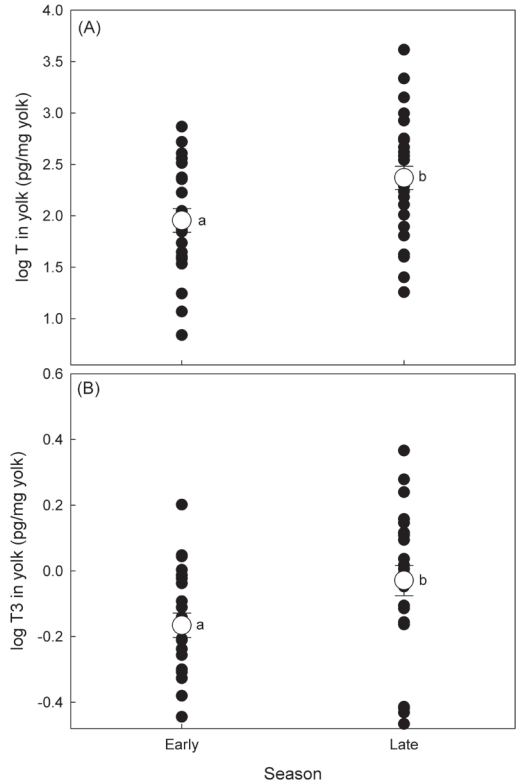


Figure 4. Concentrations of testosterone (A) and triiodothyronine (B) in yolks of Franklin's Gull (*Leucophaeus pipixcan*) eggs are higher in eggs laid later in the nest initiation period (filled circles represent observed values, open circles represent means with bars for SE, and small letters designate significantly different mean values).

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 in Franklin's Gull eggs supports the hatching asynchrony adjustment hypothesis. 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 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 ovarian tissue 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 more 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.* 2018). 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 a reflection of the seasonal and short-term dynamics of the different hormone axes in the adult females. The hypothalamus-pituitary-gonad (HPG) axis initiates activity through gonadotropin releasing hormones (GnRH) from the hypothalamus, which stimulates the release of follicle stimulating hormone (FSH) and LH from the pituitary to affect hormone secretion from the ovary.

This cascade results in changes in adult behaviors and physiology associated with reproduction on a seasonal time-scale, but also regulates short-term hormone changes associated with ovulation. In comparison, the hypothalamus-pituitary-thyroid (HPT) axis acts through thyrotropin releasing hormone (TRH) from the hypothalamus, initiating thyroid stimulating hormone (TSH) release from the pituitary, which then causes T3 to be released from the thyroid gland. This axis is also sensitive to seasonal changes associated with molting and migration (Pérez *et al.* 2018), however there is little evidence that the HPT axis exhibits shorter term changes associated with ovulation or egg production. Thyroid hormone secretion has been associated with differences in early and late nesting in the House Sparrow (*Passer domesticus*). Pre-breeding plasma T3 levels were higher in House Sparrows breeding early in the season compared to House Sparrows breeding late in the season (Chastel *et al.* 2003). Hence there could be seasonal (i.e., among-female) differences in T3 concentrations in yolk at the time of egg production. The HPG 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 HPT 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 females 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 necessary for molt regulation and migration processes and has also been shown to decrease incubation time in turtles (McGlashan *et al.* 2017). Triiodothyronine also regulates embryonic growth and differentiation of several tissues associated with the muscular, skeletal, and nervous systems (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 approximately 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.

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. More information on within and among clutch differences in hormones is needed, including information on other (i.e., non-androgen) hormones with possible links to growth and development in both embryos and chicks.

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was conducted in accordance with North Dakota State University Institutional Animal Care and Use Committee (Protocols A13056 & A16066). Eggs were collected under the authority of Federal Collection Permit 23212, and North Dakota Game & Fish Permits GNF03798718 & GNF04049484. All applicable ethical guidelines for the use of birds in research have been followed, including those presented in the Ornithological Council's "Guidelines to the Use of Wild Birds in Research" (Fair *et al.* 2010). Funding was provided by North Dakota State University. This manuscript was improved thanks to the critical review of earlier versions by J. McEvoy and two anonymous reviewers.

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