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TESTOSTERONE SECRETION IN A SOCIALLY MONOGAMOUS BUT SEXUALLY
PROMISCUOUS MIGRATORY PASSERINE

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21 Abstract

22 The steroid hormone testosterone (T) influences a multitude of traits critical to reproduction in
23 vertebrates. In birds, high male T supports territory establishment and mate attraction, but is
24 thought to interfere with parental care. Interspecific comparisons indicate that migratory species
25 with short, synchronous breeding seasons have the highest peak T, and that the seasonal profile
26 of T exhibits a rapid decline with the onset of incubation by females. We describe the T profile
27 of the migratory, socially monogamous, and biparental Eastern Kingbird (*Tyrannus tyrannus*)
28 from the high desert of eastern Oregon, USA, where breeding occurs within a short 2-3 month
29 period. Eastern Kingbirds are socially monogamous but exhibit high rates of extra-pair paternity
30 as ~60% of broods contain extra-pair young. We therefore evaluate whether Eastern Kingbirds
31 exhibit the “typical” T profile expected for a synchronously breeding migratory species, or
32 whether T is maintained at a more constant level as would be predicted for a species with
33 opportunities for mating that extend over a majority of the breeding season. Our samples were
34 divided into six periods of the reproductive cycle from territory establishment to the feeding of
35 fledglings. T did not change across stages of the nest cycle. Instead, T declined with sampling
36 date and nest density, and increased with the number of fertile females in the population. Male
37 kingbirds advertise their presence through song for most of the breeding season, and we suggest
38 that T is maintained throughout most of the breeding season because male fitness is equally
39 dependent on within- and extra-pair reproductive success.

40

41 *Keywords: androgens, hormones, kingbirds, mating system, testosterone, Tyrannus*

1. Introduction

The secretion of steroid hormones is an important proximate physiological mechanism governing the expression of many vertebrate reproductive characteristics (Adkins-Regan, 2007; Hau, 2007). In male birds, testosterone (T) is an especially important hormone because of its influence on male-male aggression and territoriality (Chandler et al., 1994; Canoine and Gwinner, 2002; Silverin et al., 2004), dominance status within groups (Peters et al., 2001), mate attraction displays such as song (Van Duyse et al., 2000; Foerster et al., 2002), mate guarding (Moore, 1984; Saino and Møller, 1995a), and possibly extra-pair mating behavior (Raouf et al., 1997; Garamszegi et al., 2005; Eikenaar et al. 2011a). T has also been shown to affect the development of sexual ornaments (Peters et al. 2006; Redpath et al., 2006; but see Owens and Short, 1995; Schlinger et al., 2008).

The stimulatory effect of T on mating behaviors can also result in the inhibition of parental behaviors (e.g., Raouf et al., 1997; Schoech et al., 1998; Peters et al., 2002) because high T males direct their activity to obtaining extra copulations, leaving less time for parental care (Oring et al., 1989; Cawthorn et al., 1998; De Ridder et al., 2000; Van Roo, 2004). Therefore, in species with male parental care, including most monogamous species, T is expected to decline rapidly prior to the commencement of paternal care (Hunt et al., 1995; Logan and Wingfield, 1995; Van Roo et al., 2003; Pinxten et al., 2007). Overall, a species' mating system is an important predictor of its seasonal T profile (reviewed by Hirschenhauser et al., 2003). For example, in polygynous species, T secretion exhibits a much slower decline throughout the nest cycle because males participate little in parental care and, instead seek additional mating opportunities (Beletsky et al., 1989). The opportunity to obtain extra-pair copulations in monogamous species (Griffith et al., 2002) should also be considered as an

important source of reproductive success and as a possible contributor to variation in testosterone among males. However, very little is known about how T secretion is modulated to balance extra-pair mating activity with parental behavior (but see Peters et al., 2001; Horton et al., 2010; Eikenaar et al., 2011b), and more attention needs to be given to monogamous species in which extra-pair fertilizations are important contributors to male reproductive success.

Peak T and T profiles (i.e., change with the reproductive cycle) also appear to vary with geographic distribution and migratory behavior. For instance, the breeding biology of many tropical birds is characterized by relatively long breeding seasons, year-round territoriality, and low breeding synchrony (Hau et al., 2008; Robinson et al., 2010; Stouffer et al., 2013). By contrast, temperate species are faced with relatively short breeding seasons, an intense period of territory establishment, and high breeding synchrony. These differences have been offered as an explanation for the generally lower peak T of tropical species compared to temperate species (Goymann et al., 2004). Similar reasoning presumably explains why migratory birds typically have higher peak T than resident species (Garamszegi et al., 2008).

A number of sources of intra-population variation in T secretion have been identified (reviewed by Kempenaers et al., 2008). Testosterone declines with time of day in many diurnal species (Bachman et al., 1987; Foerster et al., 2002; Hau et al., 2002) and with date in the breeding season, irrespective of day in the nest cycle (Hunt et al., 1995; Johnsen, 1998; Kempenaers et al., 2008). Male-male or male-female interactions can also influence the amount of T secreted by a male. As predicted by the ‘challenge hypothesis’ (Wingfield et al., 1990), aggressive interactions between males should result in an increase in circulating T. This prediction has been confirmed in some species (Moore, 1984; Wingfield, 1984; Wikelski et al., 1999; McGlothlin et al., 2007), but not in others (Van Duyse et al., 2004; Landys et al., 2007;

Landys et al., 2010). Aggressive male-male interactions are expected to increase as conspecific density increases, contributing to the positive association between T and conspecific density in some species (Wingfield and Hahn, 1994; Sasvári et al., 2009; Horton et al., 2010). Because of its role in mate attraction, T secretion also increases as a male's partner becomes fertile (Johnsen, 1998; Schwabl et al., 2005) and as the pool of fertile females within a population grows (Peters et al., 2001).

We studied individual and seasonal variation in T in Eastern Kingbirds (*Tyrannus tyrannus*) breeding in eastern Oregon, USA. Eastern Kingbirds (hereafter kingbirds) are territorial, Nearctic-Neotropical migratory passerines that breed over much of North America as socially monogamous pairs. Although biparental, females build nests and incubate eggs without male assistance. However, males do feed nestlings, though in at least some populations, females contribute more to nestling provisioning than males (Woodard and Murphy, 1999). While males do not feed the female during incubation, they take the lead in nest defense (Woodard and Murphy, 1999; Redmond et al., 2009a) and are therefore a critical component of nest success (e.g., Hayes and Robertson 1989).

Extra-pair copulations are common in kingbirds (Rowe et al., 2001) and within- and extra-pair mating success contribute equally to male reproductive success (Dolan et al., 2007). Variation in extra-pair mating success is sufficiently strong to create a significant opportunity for sexual selection (Dolan et al., 2007). Thus, this population of kingbirds provides an opportunity to examine variation in T in a migratory, socially monogamous species breeding in a highly seasonal environment where paternal care and extra-pair mating activity are likely to create conflicts between mating and paternal effort. Given the combination of migratory behavior, short breeding season, and the important role that males play in successfully raising offspring, we

might predict a high peak T early in the season followed by rapid decline during incubation (Wingfield et al., 1990). However, because extra-pair fertilizations are an important component of male kingbird reproductive success (Dolan et al., 2007), we predicted that T would not exhibit a sharp drop during incubation as seen in most monogamous species and would instead change little across stages of the nest cycle. Assuming males are sensitive to their individual social environments, we anticipated that males would respond to their competitive environment and opportunities for additional fertilizations through extra-pair relations. Consequently, we predicted that T would vary positively with nesting density and with the availability of fertile females at both local and population level scales.

2. Methods

2.1. Study area

We conducted this study from 2005 to 2009 at Malheur National Wildlife Refuge (MNWR), located near Frenchglen, Harney County, Oregon (42.817° N, 118.900° W). The study site has been described in detail elsewhere (Redmond et al., 2009b; Redmond and Murphy, 2012). Briefly, the refuge consists of a large wetland complex surrounded by dry shrub-steppe habitat. Kingbirds nest almost exclusively in willows along the watercourses that run through the refuge (Redmond et al., 2009b). Between 35 and 50 pairs of kingbirds nested within the study site each year and birds of both sexes have been color-banded at Malheur since 2002 as part of demographic and parentage studies of this population.

Kingbirds arrive at Malheur over a six-week period beginning in mid-May, but the majority arrive between the last week of May and mid-June (Cooper et al., 2009a). Nesting typically begins by mid-June and nearly all reproductive activity is finished by early to mid-

August. Beginning in mid-May, we exhaustively surveyed the study site daily to determine the identity of kingbird pairs by resighting color-banded birds. If individuals were not banded then we would attempt to capture them at some point during the season. High frequency of banded birds in the population (85-95%) coupled with observations at each nest ensured our confidence that we assigned the correct individuals to nests. Once located, nests were checked every two to three days to track the progress of females through the laying cycle so that we could determine when they were fertile. Similar to previous work on other species, we assumed that females were fertile from five days before the first egg was laid to the day the penultimate egg was laid (Birkhead et al. 1989; Birkhead and Moller 1992; Gil et al. 1999). In addition to documenting date of clutch initiation, we determined clutch size, hatching success (number of eggs to hatch), and nest success (number of young to fledge). Kingbirds raise only a single brood per year, but failed nests (a range of 55–80% fail annually) are usually replaced up through the first to second week of July, and therefore we located all replacement nests for pairs that failed. Nest locations were recorded using a Garmin GPS 72 ($\pm$ 3-4 m) and nest distances were measured with ArcMap v. 9 (ESRI, Redlands, CA, USA).

2.2. Capture and blood sampling

Kingbirds were captured using mist nets in one of two ways. First, mist nets were positioned to surround nests to capture both parents as they returned to feed nestlings during daylight hours, a passive capture technique which occurred from late June and onward. Second, throughout the breeding season we used mist nets in conjunction with a playback recording of the ritualized dawn song male kingbirds sing prior to sunrise (0230 to 0600 PST, Sexton et al., 2007) to capture males by simulating a conspecific territorial intrusion. Time to capture was

recorded as the length of time between the start of the playback and the male's capture. Once captured, kingbirds were removed immediately from the net and a blood sample (~250 uL) obtained by brachial venipuncture (usually < 5 minutes after capture). Time to capture was unknown for passive-capture adults because we had to remove ourselves from the vicinity of the nest so that parents would continue to feed young, which could potentially bias our results. To test for a possible effect, we omitted passively captured individuals and reran the analyses described below, but found no difference in the interpretation of results. Therefore, we included passively captured males in the results given below. Individuals were then banded with a unique combination of one USGS aluminum band and three colored plastic leg bands if not already banded. We measured body mass to the nearest 0.25 g with a spring scale (Pesola), tarsus and bill length to the nearest 0.05 mm (dial calipers), and wing chord and tail length to the nearest 0.5 mm (stopped wing ruler). We also measured the height and width of the cloacal protuberance with dial calipers and used these values to calculate cloacal protuberance volume ($[(0.5 * \text{width}) * \pi * \text{height}]$; Sax and Hoi, 1998).

2.3. Assays

Blood samples were kept on ice for up to 5 hours before they were centrifuged for 10 minutes. After centrifugation, we measured hematocrit (packed cell height divided by total height of cells and plasma) and then removed the plasma from the tube using a Hamilton syringe. Plasma samples were placed in microcentrifuge tubes and frozen at -20°C until assays were performed. We used a commercially available radioimmunoassay kit (ImmuChem™ Double Antibody ¹²⁵I RIA kit [MP Biomedicals #07-189102]) to quantify testosterone concentrations (T) in the plasma samples collected from male kingbirds. A parallelism test yielded parallel curves

180 between the kit's standards and serial dilutions of kingbird plasma. We did not dilute plasma
181 samples for the assays. Assays were conducted following the directions supplied by the
182 manufacturer and counts per minute (CPM) of ^{125}I labeled testosterone of each sample were
183 determined using a Wizard 1470 gamma counter (PerkinElmer, Massachusetts, USA). Plasma
184 testosterone concentrations (T) were calculated from the standard curve generated by standards
185 provided in the kit using nonlinear regression and the 'log(inhibitor) vs. response' option of
186 GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA). We conducted
187 three groups of assays: one for samples collected between 2005 and 2007 ($n = 73$), another for
188 samples collected during 2008 ($n = 53$), and a final group of assays for samples collected in 2009
189 ($n = 33$). Intra-assay and inter-assay variation were 12.8% and 7.9%, respectively. Mean T from
190 the assays conducted on samples collected between 2005 and 2007 (mean = 0.18 ± 0.051 ng/mL)
191 were significantly lower than samples assays from both 2008 and 2009 (0.37 ± 0.080 and $0.35 \pm$
192 0.104 , respectively). We found no evidence that this difference was created by any factor (date
193 of collection, day in nest cycle, or time of day) other than a quantitative difference among the
194 three assays that would potentially bias the results. One option to resolve this issue was to always
195 include a variable in analyses that distinguished among cases of the assay groups. However,
196 after preliminary analyses, we felt that this complicated results because of the addition of an
197 extra variable for which there was no biological basis. A second option was to increase T in
198 every sample from 2005-07 in a date-specific manner to incorporate the seasonal decline in T
199 that was apparent in every year (see below). We attempted this but it amplified the seasonal
200 effect beyond reasonable levels. We therefore opted for a third solution, which was to use the
201 mean differences (0.18) between samples from 2005-07 and 2008 and 2009 as a correction factor
202 that was added to every sample collected prior to 9 July in the 2005-2007 data set. We chose that

cutoff because only two samples from the 2005-2007 assays had measureable levels of T beyond 9 July ($\geq$ day 70 in Fig. 1A).

2.4. Statistical analyses

To assess the efficacy of our measures of T, we regressed $\log_{10}$ transformed cloacal protuberance volume against T to test the prediction that physiologically meaningful values of T should be apparent as a positive relationship between the two variables. To test for an effect of playback on T we first compared T between males captured passively and those captured using playback with an analysis of variance (ANOVA), and analysis of covariance (ANCOVA) with date included as a covariate. Because variable amounts of time were required to capture males using playback (range = 20 sec to 159 min) we also tested for an effect of length of playback on T by regressing T against log-transformed values of time to capture with the expectation that a positive relationship would emerge if playback influenced T secretion.

We described the breeding season profile of T by comparing T among six stages of the reproductive cycle using ANOVA. The six stages were: (1) territory establishment (males on a territory but without a mate, or males caught on the territory of another male), (2) pre-laying (male was paired, a nest had been started, but female had not yet begun to lay), (3) egg laying (clutch begun but not yet completed), (4) incubation (female incubating eggs), (5) nestling care (parents feeding dependent young in nest), and (6) post-fledging care (young out of nest and still under parental care). Some individuals of both sexes never establish territories or pairbonds and appear to remain in a “floater” population (Cooper et al. , 2009b). We assumed males caught on the territory of another male during a dawn song period were unpaired “floaters” (Cooper et al.,

2009b) because prior experience had shown that territory holding males are virtually always on their territory during the dawn song period (Sexton et al., 2007).

We used multiple regression analysis to test simultaneously for the contribution of different predictor variables to variation in T in kingbirds. Our expectation was that T would vary temporally, declining over the course of the morning and as the breeding season progressed. Time of day was measured as minutes from 0000 hr PST, while date of capture was measured continuously from May 1 (= day 1). We also anticipated that T would vary with each male's social environment, and predicted an increase in T with increasing nesting density and availability of fertile females. Conspecific nesting density was measured as the inverse of the distance from a captured male's nest to the nearest kingbird neighbor's nest (nearest neighbor distance) and mean of the three nearest kingbird nests (neighbor density). The rationale for our use of the three nearest neighbors was partly to be consistent with our previous work (Sexton et al. 2007), and partly a consequence of the geometry of our study site. Nearly all pairs were arrayed linearly along the river running through MNWR, and the use of the three nearest neighbors would likely include the neighbors up- and downstream from the focal bird, plus one other. Given the linear arrangement of pairs, it seemed to us that including more males would expand the zone of regular interactions of males to an unrealistic level because with three the mean distance of them to the focal bird was already 1 km (see below). We used two measures of fertility to determine if T was influenced by females outside of the pair bond. Local fertility was defined as the number of females from the three nearest neighbors that were fertile on the day of the male's capture. However, because distance to a female kingbird's extra-pair mate averaged 1.8 km and was as great as 15 km (Dolan et al., 2007), we included the total number of fertile females available in the study population as population fertility. Given the nature of the

information we used, there was potential for multicollinearity among certain variables. We acknowledged this issue and checked variance inflation factors (VIF) for all independent variables. Nearest neighbor distance and the mean distance to the three nearest neighbors had the highest VIF at 4.8 and 4.7, respectively. All other variables had $VIF < 1.8$, indicating multicollinearity was not a problem.

We used an information theoretic approach to calculate AIC values corrected for small sample size (AIC_c) to evaluate model fit. All models within 4 AIC_c units of the top model were considered competitive unless they contained uninformative variables (Arnold, 2010). We then calculated ΔAIC_c , model probabilities, and model weights. Model-averaged parameter estimates of regression coefficients and standard errors were calculated (Burnham and Anderson, 2002) to determine if variables were important predictors of T. Variables whose parameter estimate's 85% confidence interval (Arnold, 2010) did not include zero were deemed to have contributed to variation in T.

Date and time of sample collection, and population level fertility were known for all captured individuals ($n = 101$) and we used this sample to describe variation in T across stages of the nesting cycle. However, we had 17 samples from non-paired males, which, by definition, did not have a nest. Therefore, to comply with requirements of a standard information theoretic approach, we only used samples from paired individuals with complete information on nest density and female fertility in the analysis ($n = 81$). A subset of males were caught more than once throughout the entire study, thus we randomly chose one sample per male so each individual was represented by a single sample in the analysis. T in the subset of males that were captured two or more times in one year were compared using paired t-tests to evaluate whether an individual male's T changed between sampling dates. Values for T, population fertility, and

local fertility did not conform to a normal distribution and were $\log_{10}$ transformed. Statistical significance was set at $P = 0.05$ and statistics are presented as means $\pm$ SE or regression coefficients $\pm$ SE, and sample size (n). Analyses were conducted in Statistix 9.0 (Analytical Software, 2008) and SPSS 11.5 (SPSS Inc., 2002).

3. Results

3.1. *Testosterone concentration in kingbirds*

Plasma T levels ranged from undetectable to a maximum of 2.89 ng/mL (mean = 0.28 ± 0.043 ng/mL, $n = 101$). Levels of T in the group of assays that were done for samples collected between 2005 and 2007 (mean = 0.20 ± 0.047 ng/mL, $n = 38$) were lower ($F = 11.97$, $P < 0.001$) than the assays for the samples collected in 2008 (mean = 0.31 ± 0.064 ng/mL, $n = 40$) and 2009 (mean = 0.39 ± 0.138 ng/mL, $n = 23$). As confirmation that variation in T among males represented physiologically relevant differences, cloacal protuberance volume and T were positively and significantly related ($\beta = 0.094 \pm 0.0317$, $R^2 = 0.105$, $n = 57$, $P = 0.004$).

To test for a possible effect of the capture methods we used, we compared T between passively captured individuals and those captured using playback. Mean T of kingbirds captured using playback (0.30 ± 0.049 , $n = 86$) was higher than that of those captured passively (0.13 ± 0.069 , $n = 15$; $F = 29.47$, $P < 0.001$). However, capture date (playback: 25 June $\pm$ 1.89 days; passive: 15 July $\pm$ 3.33 days; $t = 4.31$, $P < 0.001$) and time of day at capture (playback: 0457 $\pm$ 0038 hr PST; passive: 0844 $\pm$ 0203 hr PST; $t = 14.23$, $P < 0.001$) differed between playback and passively captured males, and T varied with both date and time of capture (see below). Inclusion of date and time of capture as covariates in the comparison of T between playback and passive capture males eliminated the effect of capture method (date: $F = 34.85$, $P < 0.001$; time: $F = 1.17$,

$P = 0.282$; capture method: $F = 1.29$, $P = 0.259$). T was also not influenced by the length of time required to capture the bird as there was no relationship between T and time elapsed between the start of playback and capture ($\beta = -0.002 \pm 0.00047$, $F = 0.18$, $P = 0.672$, Fig. 2).

3.2. Testosterone profile of kingbirds

Plasma testosterone levels differed among reproductive stages (ANOVA: $F = 11.24$, $P < 0.001$, Fig. 3). T was highest during territory formation and **did not decline until** dependent young were in the nest. T did not differ between the nestling and fledging stage, and T from samples collected during the fledging stage did not differ from the laying and incubation stages, probably because of high variability in T among males during the laying and fledgling stages. We obtained two or more blood samples within a year for 16 males (Fig. 4). Time elapsed between samples for these individuals ranged from 10 to 51 days (mean = 22.0 ± 3.04 days). T declined between the first and second samples for 11 males (mean difference = -0.48 ± 0.221), increased in four (mean difference = 0.39 ± 0.397), and was unchanged for one. Counting the male whose T was unchanged as a decline, a Fisher's Exact Test indicated that the pattern of change (12 decline and 4 increase) did not differ from that expected by chance ($P = 0.140$ for a one-tailed test based on an expected decline). Two males were sampled three times within a year and for both of these individuals T increased between the first and second sample, and for one of the males the third sample had a higher T than the first blood sample. A paired t -test confirmed that T did not differ between the first and second samples of the 16 males (mean = 0.23 ± 0.184 ng/uL, $t = 1.25$, $P = 0.231$).

3.3. Variation in T: temporal influences and social environment

Sampling dates encompassed essentially the entire kingbird breeding season at MNWR (28 May to 8 August). T was highest from the early to middle breeding season, and from roughly the end of the first week of July onward T dropped to very low levels ($\beta = -0.048 \pm 0.0060$, $R^2 = 0.451$, $F = 64.83$, $P < 0.001$, Fig. 1A). Nearly all capture attempts were in the morning (0358 to 0900 hrs PST), and T declined with capture time ($\beta = -0.006 \pm 0.0011$, $R^2 = 0.258$, $F = 27.50$, $P < 0.001$, Fig. 1B). Distance to the nearest neighbor (673 ± 179.1 m, median = 289, range = 45 to 12,163 m, $n = 81$) and mean distance to the three nearest neighbors varied widely ($1,005 \pm 191.4$ m, median = 543, range = 141 to 12,718 m, $n = 81$). In a univariate regression, T was unrelated to both nearest neighbor distance ($\beta = 0.032 \pm 0.3229$, $F = 0.10$, $P = 0.922$) and neighbor density ($\beta = -0.254 \pm 0.3891$, $F = 0.43$, $P = 0.516$; Fig. 5A). Local fertility on the day of sample collection ranged from 0 to 3 (mean = 0.5 ± 0.09 , $n = 81$) while population fertility ranged from 0 to 16 (mean = 4.1 ± 0.49 , $n = 81$). T exhibited positive associations with both local fertility and population fertility in separate univariate analyses (local: $\beta = 0.427 \pm 0.1641$, $R^2 = 0.079$, $F = 6.76$, $P = 0.011$; population: $\beta = 0.126 \pm 0.0291$, $R^2 = 0.193$, $F = 18.85$, $P < 0.001$, Fig. 5B).

We used an information theoretic approach to determine how temporal and social variables affected T in multivariate relationships. The top model from the regression analyses included date and capture time, along with population fertility and neighbor density (Table 1). However, because of considerable model uncertainty we used model averaging to determine which variables best explained variation in T among males. Confidence intervals of model-averaged parameter estimates for date, population fertility, and neighbor density did not include zero, and indicated that T declined over the course of the breeding season and as neighbor density increased, and increased as population fertility increased. The parameter estimate for time of capture suggested a probable decline in T over the course of day, but T was independent

of local fertility and nearest neighbor distance (Table 2). Together, capture date, population fertility, and neighbor density accounted for nearly 60% of the variation in T ($R^2 = 0.584$, $F = 35.97$, $df = 3, 77$, $P < 0.001$).

Date and nest stage were strongly correlated (Spearman rank correlation = 0.814, $n = 81$, $P < 0.001$), and the decline of T with date in the multiple regression was conceivably driven by progressive transitions through nest cycle stages. We therefore simultaneously tested for associations between T and both date and nest stage by entering date, population fertility, and neighbor density in a general linear model as covariates with nest stage as a categorical variable. All variables contributed significantly to differences in T (Table 3). T declined with date ($\beta = -0.058 \pm 0.0095$, $F = 6.17$, $P < 0.001$), neighbor density ($\beta = -0.759 \pm 0.2631$, $F = 2.89$, $P = 0.005$), and increased with population fertility ($\beta = 1.017 \pm 0.2379$, $F = 4.28$, $P < 0.001$). T did not differ among males at any stage between pre-laying and the nestling period when we accounted for the three other variables (Fig. 3). The significance associated with nest stage was a consequence of unexpectedly high T among males caring for fledglings (Tukey's test). Reanalysis after restricting the sample to males from the pre-laying through nestling stages indicated T was unrelated to nest stage ($F = 1.20$, $df = 3, 69$, $P = 0.317$), but that date, population fertility, and neighbor density were all associated significantly with T (all P 's < 0.011).

4. Discussion

Eastern Kingbirds are socially monogamous, long distance migratory passerines that have a relatively short breeding season at MNWR (Cancellieri and Murphy, 2013). Among many passerines, these properties are associated with a peak in T early in the breeding season, followed by sharp declines as a male's mate initiates incubation (Hunt et al., 1995; Van Roo et al., 2003;

Pinxten et al., 2007; Stanley et al., 2011). Contrary to expectations, T remained relatively constant across stages of the nest cycle. Intra-individual comparisons also suggested that T showed no consistent pattern of decline between first and later samples for males captured ≥ 2 times per season. However, independent of stage of the nest cycle, T declined with date and over the course of the day. Finally, social factors were important contributors of T variation as the secretion of testosterone increased as the number of fertile females increased in the population but, contrary to our predictions, was lower in areas of high nest density.

4.1. Testosterone profile of Eastern Kingbirds

Conventional wisdom (Wingfield et al., 1990) is that T of monogamous species is high early in the breeding season to support territory establishment and mate attraction, but that T then declines rapidly during incubation to facilitate the transition of males to parental care (e.g., Hunt et al., 1995; Van Roo et al., 2003; Pinxten et al., 2007; Stanley et al., 2011). However, recent studies suggest that high T and paternal care are not necessarily incompatible (Townsend et al., 1991; Wynne-Edwards and Timonin, 2007; Neff and Knapp, 2009). In the double-brooded North American breeding Barn Swallow (*Hirundo rustica*), for instance, Eikenaar et al. (2011a) showed that male T did not decline between the female fertile period and incubation, and that male feeding effort was independent of T. T-implanted Great Tits (*Parus major*) also maintained nestling feeding rates as high as controls (Van Duyse et al., 2000). Our results are similar to the temporal patterns exhibited by Barn Swallows as T was equally high from the pre-breeding phase through the end of nestling feeding (Fig. 3), and when extrinsic factors were accounted for, T did not decline across phases of the nesting cycle. The absence of a decline in T at incubation is found in polygynous species (e.g., Beletsky et al., 1989), and an increasing number of socially

monogamous species such as Superb Fairy-wrens (*Malurus cyaneus*; Peters et al., 2001), European Stonechats (*Saxicola torquata*; Schwabl et al., 2005), Dark-eyed Juncos (*Junco hyemalis*; Jawor et al., 2006), Orange-crowned Warblers (*Oreothlypis celata*; Horton et al., 2010) and Barn Swallows (Eikenaar et al., 2011a). Experimentally elevated T inhibits paternal behavior (Saino and Møller, 1995b; Schoech et al., 1998; De Ridder et al., 2000; de la Cruz et al., 2003), but individually maintained endogenous male T does not appear to inevitably inhibit full expression of parental care in males of many species (see above). Maintenance of T levels could possibly be related to an increased chance that males sire more young, which, for socially monogamous species, would be via attracting females outside of the pair bond thus gaining extra-pair fertilizations (see “Social factors” below).

Courtship display (Wiley and Goldizen, 2003) and song production (Ketterson and Nolan, 1992; de Ridder et al., 2000; Van Duyse et al., 2000) are testosterone-dependent in birds. Male kingbirds with dependent young still participate daily in dawn songs well into July (Sexton et al., 2007). We suggest this is a result of the prolonged secretion of T that we observed. Song appears to be a cue used by female kingbirds to assess male quality (Murphy et al., 2008) as the earliest and most vigorously singing males have the greatest extra-pair success in kingbirds (Dolan et al., 2007). Prolonged secretion of T by male kingbirds should thus increase reproductive success.

Relatively little is known about diel variation in T in birds, but our results agree with the few other published studies in suggesting that it declines over the course of the day (Bachman et al., 1987; Foerster et al., 2002; Hau et al., 2002; but see Schwabl et al., 2005; Peters et al., 2006). Why this is so is not fully understood (Kempnaers et al., 2008), but high T early in the day is possibly favored by anticipation of or actual participation in male-male aggression and mate

attraction early in the day when the female's "fertilization window" occurs (Williams, 2012). Kingbird copulations appear to be limited almost entirely to the pre-dawn darkness as over 40+ combined years of extensive field experience with kingbirds by Murphy and collaborators has yielded only three observed copulations. If T acts to inhibit paternal care (which it may not), a circadian pattern of testosterone secretion may be the mechanism that allows males to avoid conflicts between mating and paternal behavior. Higher T in the predawn darkness may stimulate dawn song as a means of pursuing extra-pair copulations. The decline following this time may then ensure that parental behaviors are expressed to promote within-pair nest success.

4.2. Social aspects of testosterone variation

Our detection of an inverse relationship between T and conspecific density is, to our knowledge, unique in that other studies have either reported no relationship (Eikenaar et al., 2011b) or an increase in T under conditions of high density (Wingfield and Hahn, 1994; Sasvári et al., 2009). The latter is the expectation under the assumption that T mediates aggression and that male-male conflict increases when nesting density is high (Wingfield et al., 1990). The relationship between T and aggression as mediated through male-male interaction is often studied by using simulated territorial intrusions (STI) that mimic aggressive interactions between males. STI typically succeed in generating aggressive behavioral responses from focal males (e.g., Schwabl et al., 2005; McGlothlin et al., 2007; Gill et al., 2008), but the expected rise in T following STI does not always materialize (Wingfield and Hahn, 1994; Silverin et al., 2004; Busch et al., 2008; Gill et al., 2008; this study) and has even been found to lead to a decrease in T (Van Duyse et al., 2004; Peters et al., 2006; Landys et al., 2007).

On the other hand, almost all studies in which testosterone was artificially elevated by T-implants showed that territory size of implanted males increased, which led to decreases in density (e.g., Wingfield, 1984; Chandler et al., 1994; Moss and Lambin, 1994; Alonso-Alvarez and Velando, 2001; but see Chandler et al., 1997). This same mechanism may have generated the inverse relationship we detected between T and nesting density in kingbirds; males with naturally high T may held larger territories. However, given that ours was a non-experimental study, it is equally likely that males with naturally high T selected territories with few neighbors, whereas low T males with, presumably greater tolerance, settled in high density areas. The failure of STI to elicit an increase of T in kingbirds is consistent with the detection of low T among males nesting at higher density. Short-term aggressive interactions among kingbird males nesting in close proximity to one another probably do not elevate T.

The importance of T in regulating reproductive behavior of vertebrates is unquestioned (Adkins-Regan, 2007; Hau, 2007). Male T is generally at or near its annual peak when the mates of males of monogamous species are fertile (e.g., Schwabl et al., 2005; Pinxten et al., 2007). Males of some monogamous (Peters et al., 2001) and polygynous species (Johnsen, 1998) have also been shown to track female fertility at larger spatial scales. Maintenance of relatively constant T by kingbirds throughout much of the nest cycle and a strong positive association between T and population level fertility indicates that male kingbirds are responsive to the availability of fertile females at large temporal and spatial scales.

Among socially monogamous species, the potential for multiple broods and/or opportunities for extra-pair fertilizations (Griffith et al., 2002) are likely to favor maintenance of physiologically relevant levels of T. For instance, males of the socially monogamous Superb Fairy-wren maintain T through the nestling period (Peters et al., 2001), pairs raise multiple

454 broods (Cockburn et al., 2003) and exhibit possibly the highest rates of extra-pair paternity
455 known at 76% (Mulder et al., 1994). Similarly, Barn Swallows regularly raise two broods per
456 year, male T varies little between the incubation and nestling periods, and nearly a third of Barn
457 Swallow young are of extra-pair origin (Eikenaar et al., 2011a). In both species, fitness payoffs
458 exist for males that maintain sexual activity throughout the nest cycle. Eastern Kingbirds are
459 single brooded, but in virtually all years > 50% of nests fail, and most females replace failed first
460 nesting attempts if failure occurs before mid-July (Murphy, 1996; Cooper et al., 2011). As a
461 consequence, fertile females are available throughout most of the kingbird breeding season,
462 males advertise their availability through dawn song well into July (Sexton et al., 2007), and
463 every year 60% ($\pm 5\%$ SE) of kingbird nests contain extra-pair young (Rowe et al., 2001; Dolan
464 et al., 2007). Tree Swallows (*Tachycineta bicolor*) also exhibit extremely high rates of extra-pair
465 paternity (Whittingham and Dunn, 2014), but unlike the aforementioned species, exhibit a
466 distinct drop in T between the early nest cycle and incubation (Stanley et al., 2011). Tree
467 Swallows are single brooded, but unlike kingbirds, experience far less nest failure and therefore
468 the decline in T may reflect a greatly reduced opportunity for males to encounter fertile females
469 after the initial phase of egg-laying. The responsiveness of male kingbirds to fertile females on
470 the population but not local level also follows from the spatial scale over which extra-pair
471 fertilizations occur. Dolan et al. (2007) showed that nearly 50% of extra-pair sires in this
472 population were not nearest neighbors. Instead, extra-pair partners were separated by an average
473 distance of 1.8 km, nearly twice the average distance to the three nearest neighbors upon which
474 local fertility was calculated (1,005 m). Circumstantial evidence suggests that female kingbirds
475 travel to extra-pair male locations, and male kingbirds may respond to population-wide changes
476 in female fertility by maintaining T to be prepared to enhance reproductive success via extra-pair

fertilizations. Much of the interspecific variation in male T among species (see Garamszegi et al., 2008) may thus relate as much to the availability of fertile females over extended periods of the breeding season as to the potential for aggressive interactions among territory holding males.

4.3. Conclusion

Our analysis of breeding season variation in T of Eastern Kingbirds causes us to question the advisability of viewing patterns of T in socially monogamous male birds solely from the confines of the need to establish a territory to support reproduction by a future mate. The view that testosterone is incompatible with full expression of paternal care is eroding, and given the widespread occurrence of extra-pair mating opportunities in many species (Griffith et al., 2002), selection for maintenance of male reproductive capacity beyond his mate's fertile period seems as likely to influence patterns of testosterone production as male-male aggression or selection for parental behavior. T-implant studies demonstrate that increased T typically elevates aggression and interferes with parental behaviors (see above), but testosterone's role in modulating aggression is increasingly viewed as "permissive"; a little goes a long way and other mechanisms may fine-tune male aggression (Schwabl et al., 2005; Wingfield 2012). Although more manipulative studies on species that have not been previously studied would be useful, we suggest that more studies that evaluate and control for the potential sources of natural variation in T would be equally useful in furthering our understanding of the role that T plays in eliciting mating and parental behavior. Given the very high rate of extra-pair paternity of kingbirds (Rowe et al., 2001; Dolan et al., 2007), we suggest that the seasonal T profile of kingbirds has been mainly influenced by the extra-pair mating system, and we expect this to be true of other species

in which mating opportunities exist outside of the pair bond for an extended portion of the breeding season.

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Table 1. Results of model selection from the regression analysis of variation in testosterone concentration [T] of Eastern Kingbirds breeding at Malheur National Wildlife Refuge, Oregon, in relation to date (Date) and time (Time) of sample collection, availability of fertile females at a local (LocFert) and population level (PopFert), nearest neighbor distance (NND1), and neighbor density (NND3). Statistics include Akaike Information Criterion (corrected for small sample size; AIC_c), difference in AIC_c from the top model (ΔAIC_c), the likelihood and weight of each model, and number of parameters (K). For all models, $n = 81$.

Model	AIC_c	ΔAIC_c	Likelihood	Weight	K
Date, Time, PopFert, NND3	-22.176	0	1	0.2014	6
Date, PopFert, NND3	-22.061	0.115	0.9441	0.1902	5
Date, Time, PopFert, NND3, LocFert	-20.752	1.424	0.4907	0.0988	7
Date, PopFert, NND3, LocFert	-20.372	1.804	0.4058	0.0817	6
Date, Time, PopFert, NND1	-20.195	1.981	0.3714	0.0748	6
Date, PopFert, NND3, NND1	-19.93	2.246	0.3253	0.0655	6
Date, Time, PopFert, NND3, NND1	-19.895	2.281	0.3197	0.0644	7
Date, PopFert, NND1	-19.338	2.838	0.242	0.0487	5
Date, Time, PopFert	-19.01	3.166	0.2054	0.0414	5
Date, Time, PopFert, NND1, LocFert	-18.901	3.275	0.1945	0.0392	7
Date, Time, PopFert, LocFert	-18.638	3.538	0.1705	0.0343	6
Date, Time, PopFert, DenNeigh, DenLoc, LocFert	-18.464	3.712	0.1563	0.0315	8
Date, PopFert, NND3, NND1,	-18.244	3.932	0.14	0.0282	7
Intercept only	42.259	64.435	-	-	2

Table 2. Model averaged parameter estimates of variation in testosterone concentration of Eastern Kingbirds in relation to date (Date) and time (Time) of capture, availability of fertile females at a population and local level, and nearest neighbor distance (NND1) and neighbor density (NND3). Data (n = 81) are for birds breeding at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009. Model averaged estimates include the coefficient $\pm$ SE, along with 85% confidence interval surrounding the regression coefficients.

Variable	Regression coefficient	85% Confidence Interval	
		Lower	Upper
Date	-0.042 ± 0.0063	-0.0510	-0.0330
Time	-0.001 ± 0.0008	-0.0022	0.0001
Population fertility	0.936 ± 0.2580	0.5649	1.3078
NND3	-0.533 ± 0.3159	-0.9881	-0.0783
NND1	-0.033 ± 0.2203	-0.3500	0.2844
Local fertility	-0.188 ± 0.3541	-0.6985	0.3215

Table 3. Analysis of variation in testosterone concentration ($n = 81$) from Eastern Kingbirds breeding at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009 using general linear models with sample date, population level fertility, and neighbor density included as covariates and nest stage as a categorical variable.

Source	df	SS	$F (P)$
Nest stage	4	8.903	3.60 (0.001)
Date	1	23.544	38.12 (< 0.001)
Population fertility	1	11.288	18.28 (< 0.001)
Neighbor density	1	5.140	8.32 (0.005)
Error	73	45.084	

Figure legends

Fig. 1. Testosterone concentration (T; ng/ml, $n = 81$) in Eastern Kingbirds sampled at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009 in relation to (A) date of sample collection where 1 May = 1 ($\log_{10}[T]$ vs. date, $P < 0.001$), and (B) time of day when samples were taken, where time is measured in minutes from midnight ($\log_{10}[T]$ vs. time, $P < 0.001$).

Fig. 2. Testosterone concentration (T; ng/ml, $n = 81$) in Eastern Kingbirds captured by simulated territorial intrusion at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009 in relation to time taken to capture the individual ($\log_{10}[T]$ vs. time to capture, $P = 0.672$).

Fig. 3. Mean testosterone concentration across stages of the reproductive cycle of Eastern Kingbirds breeding at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009. Error bars represent one SE, while actual mean, SE (in parentheses), and sample sizes at each stage are given above the bars.

Fig. 4. Change in testosterone concentration between first and second sample periods for male Eastern Kingbirds captured twice in a year ($n = 16$) at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009. Lines connect first and second sample periods for each male.

Fig. 5. Testosterone concentration of male Eastern Kingbirds ($n = 81$) breeding at Malheur National Wildlife Refuge, Oregon, between 2005 and 2009 in relation to (A) nesting density ($\log_{10}[T]$ vs. nesting density, $P = 0.516$) and (B) number of fertile females available in the

population ($\log_{10}[T]$ vs. number of fertile females, $P < 0.001$). Nesting density was measured as $\log_{10}$ of the inverse of the mean distance to the three nearest kingbird nests.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5