

1 **Title: Inter-annual stability and age-dependent changes in plasma testosterone levels in a**  
2 **longitudinally monitored free-living passerine bird**

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32 **Abstract:**

33 While seasonal trends in the testosterone-driven hormonal regulation of resource allocation are known  
34 from cohort population samples, data on the inter-annual individual stability of blood plasma  
35 testosterone levels in wild birds are lacking, and our understanding of age-dependent changes is  
36 limited. We assessed plasma testosterone levels in 105 samples originating from 49 repeatedly  
37 captured free-living great tits (*Parus major*) to investigate their relative long-term stability and  
38 lifetime changes. Furthermore, we examined the inter-annual stability of selected condition-related  
39 traits (carotenoid- and melanin-based plumage ornamentation, ptilochronological feather growth rate,  
40 body mass, and haematological heterophil/lymphocyte ratio) and their relationships to testosterone  
41 levels. We show that testosterone levels in both sexes are inter-annually repeatable, both in their  
42 absolute values and individual ranks (indicating the maintenance of relative status in a population), yet  
43 with higher stability in females. Despite this stability, in males we found a quadratic dependence of  
44 testosterone levels on age, with a peak in midlife. In contrast, female testosterone levels showed no  
45 lifetime trend. The inter-annual stability of condition-related traits was mostly moderate and unrelated  
46 to plasma testosterone concentrations. However, males with elevated testosterone had significantly  
47 higher carotenoid-pigmented yellow plumage brightness, presumably serving as a sexually selected  
48 trait. Showing inter-annual stability in testosterone levels, this research opens the way to further  
49 understanding of the causes of variation in fitness-related traits. Based on a unique longitudinal  
50 dataset, this study demonstrates that male plasma testosterone undergoes age-related changes that may  
51 regulate resource allocation. Our results thus demonstrate that male birds undergo hormonal  
52 senescence similar to mammals.

53

54 **Keywords:** testosterone, condition-related traits, passerine birds, ageing, senescence, immunity,  
55 ontogeny, repeatability.

56

57 **Introduction**

58 Testosterone (T) serves as a key endocrine mediator regulating the balance between investments into  
59 reproduction and various condition-related traits involved in self-maintenance (Fusani, 2008; Hau &  
60 Goymann, 2015; Kempenaers, Peters, & Foerster, 2008). Therefore, T levels may reflect individual  
61 condition, with links to immune function, physical activity, metabolism, growth, reproductive  
62 behaviours, and the expression of sexually selected ornamental traits (Hau and Goymann 2015). In  
63 bird populations, striking variations in T levels exist between different individuals, sexes, and  
64 throughout seasons. For example, in the blue tit (*Cyanistes caeruleus*) a ca. 200-fold difference exists  
65 between males with the highest and lowest T levels (Kempenaers et al., 2008). However, little is  
66 currently known about the individual inter-annual stability of T levels and T age-dependent trends in  
67 birds.

68 Avian T levels mirror many intrinsic and extrinsic factors (Kempenaers et al., 2008). Males have  
69 higher T levels than females, especially during breeding when T controls traits linked with mating,  
70 territorial aggressiveness, and parental care (Hau & Goymann, 2015). In temperate birds, male T  
71 levels peak with the increasing photoperiod during the early breeding season, followed by a decrease  
72 in the feeding period, after which T drops to steadily low levels (e.g. Van Duyse et al. 2003). Although  
73 less well understood, female plasma T follows a similar trajectory with lower amplitude and with a  
74 peak corresponding to the time of ovulation (Ketterson, Nolan, & Sandell, 2005). Importantly,  
75 variation in T levels reflects social status (Hau & Goymann, 2015), which is manifested through  
76 sexually selected condition-dependent carotenoid- (Albrecht et al. 2009; Svobodová et al. 2013) and  
77 melanin-pigmented ornaments (Griffith, Parker, & Olson, 2006; Guindre-Parker & Love, 2014).

78 Given the links between individual quality and T levels, long-term T stability in intra-population  
79 ranking can be predicted. A lack of T inter-annual stability would result in between-years variations in  
80 the related quality components. To our knowledge, studies specifically testing avian plasma T  
81 stability between years are presently lacking. Even in mammals, with more longitudinal data on  
82 repeatedly measured individuals (e.g. Perret 1992; Harman et al. 2001; Bernstein et al. 2012; Wolf et

83 al. 2018), interannual T stability has only been rigorously tested in humans (Burger et al., 2000) and a  
84 few other mammals (Montano, Robeck, Steinman, & O'Brien, 2017; Perret, 1992).

85 After an initial increase of T during adolescence in both sexes, a higher amplitude of changes  
86 throughout ontogeny with a decrease during senescence is expected in males (Chahal & Drake, 2007).  
87 In mammals, this trajectory of hormonal senescence has been found in both cross-sectional and  
88 longitudinal studies, e.g. in humans (Harman et al., 2001; Vermeulen, Rubens, & Verdonck, 1972),  
89 macaques (*Ateles geoffroyi*; Hernández-López, Cerda-Molina, Díaz-Díaz, Chavira-Bolaños, &  
90 Mondragón-Ceballos, 2012) and beluga whales (*Delphinapterus leucas*; Montano et al., 2017).  
91 Although a similar pattern may be predicted also for birds, current evidence for age-related changes in  
92 avian T levels is mixed. A single study used longitudinal data which revealed a peak for middle-age  
93 birds and a subsequent age-related decline in T levels in the Florida scrub-jay (*Aphelocoma*  
94 *coerulescens*; Wilcoxon, Bridge, Boughton, Hahn, & Schoech, 2013). In all other (cohort) studies, age-  
95 related decreases in T levels have only been detected in the Japanese quail (*Coturnix japonica*; Balthazart  
96 et al. 1984), the common tern (*Sterna hirundo*; Nisbet et al. 1999), the pied flycatcher (*Ficedula*  
97 *hypoleuca*; Moreno et al. 2014) and the barn swallow (*Hirundo rustica*; Adámková et al. 2019). In  
98 contrast, a majority of avian studies based on comparisons of young vs adult individuals (Belthoff et  
99 al. 1994; Schoech et al. 1996; Madsen et al. 2007) or cross-sectional data (Smith et al. 2005; Peters et  
100 al. 2002) reported no effect of age on male T levels. These results suggest inconsistency in age-  
101 dependent T changes in birds. Furthermore, little attention has been paid to female T levels despite  
102 their potentially non-negligible role in female physiology (Goymann & Wingfield, 2014) but see  
103 Moreno et al. (2014). Thus, longitudinal studies in free-living birds of both sexes investigating  
104 relationships between T age-related changes and fitness-related traits are lacking, but highly needed  
105 (Kempnaers et al., 2008).

106 In this article, we ask (1) whether plasma T levels in birds are inter-annually stable, and (2) if they  
107 undergo any ontogenetic changes. In a longitudinally monitored free-living great tit (*Parus major*)  
108 population, we obtained plasma samples and related data from 49 individuals of both sexes re-  
109 captured 2-3 times in subsequent years (2012-2017). Firstly, assuming links to individual quality, we

110 predicted high inter-annual repeatability in individual T ranks, especially in males (Kempnaers et al.,  
111 2008). Secondly, assuming effects of ontogenetic development and senescence, we predicted age-  
112 dependent changes in plasma T levels with a maximum in midlife (Bouwhuis, Sheldon, Verhulst, &  
113 Charmantier, 2009). Thirdly, assuming life-history trade-offs mediated by T, we predicted negative  
114 associations between age-related T levels and condition-related traits (body mass and  
115 ptilochronologically measured feather growth rates, FGR), reflecting the organismal nutritional status  
116 (Grubb, 2006), but assumed a positive relationship between T levels and actual physiological stress  
117 (e.g. Deviche et al., 2014) assessed through haematological traits (heterophil: lymphocyte ratio, H/L  
118 ratio; Davis et al. 2008) on the one hand, and sexually selected ornaments (melanin-pigmented black  
119 breast stripe area and carotenoid-based plumage coloration (Hegyi, Szigeti, Torok, & Eens, 2007;  
120 Quesada & Senar, 2006) on the other. Finally, in T-dependent traits, we predicted inter-annual stability  
121 linked to T levels.

## 122 **Materials and methods**

### 123 **Field procedures**

124 A total of 886 adult free-living great tits were captured during breeding seasons (April–May) between  
125 2012 and 2017 in a deciduous forest at the edge of Prague, Czech Republic (for a detailed description  
126 of the study locality and field procedures, see Supplementary materials and methods online 1; SMMO  
127 1 in SI), in total 49 individuals (28 males and 21 females) and repeatedly sampled for blood plasma  
128 (for age-structure see Fig. S1). The study area is located near the Kbely meteorological station (Czech  
129 Hydrometeorological Institute), where information on mean air temperature seven days before  
130 capturing (further referred to as temperature) was obtained. To standardise sampling, all birds were  
131 captured into a mist net when their nestlings were 7-14 days old. Immediately after capture,  
132 approximately 150  $\mu$ l of blood was collected from each individual by an insulin syringe (Omicron 50-  
133 50I.U./0,5ML 30G x12; B. Braun, Melsungen, Germany) from a jugular vein. Two blood smears per  
134 individual were prepared from drops of blood, part of the sample was stored frozen in a microtube  
135 with 96% ethanol for later use (not used in this study) and a subsample of the blood (ca. 70-100  $\mu$ l)  
136 was stored in a cool box and transported later the same day into the laboratory. Each blood sample was

137 centrifuged in a microcentrifuge (type 5424, Eppendorf, Hamburg, Germany) at 8000 rpm for 5  
138 minutes and the separated plasma was frozen at  $-80^{\circ}\text{C}$ . In the field, weight (measured by a digital  
139 scale,  $d = 0.02$  g, type PPS200, Pesola, Schindellegi, Switzerland) and tarsus length (measured by a  
140 digital calliper, accuracy 0.01 mm; Kinex, Prague, Czech Republic) were recorded to later calculate  
141 the size-standardised body mass as the ratio of weight to tarsus length (further referred to as body  
142 mass). The second outermost tail rectrix from the left side was collected from each individual for the  
143 ptilochronological assessment (Grubb, 2006). To assess melanin-based and yellow carotenoid-based  
144 plumage ornamentation, we first collected a standardised digital image of the black breast stripe using  
145 a scanner (type Perfection V30, Seiko Epson, Nagano, Japan; Bauerová et al., 2017). Then, samples of  
146 ornamental feathers from the upper part of the yellow breast area were collected for later light spectral  
147 analysis (ca. 20-25 feathers). Finally, each individual was tagged with a steel ring with a unique code  
148 of the Czech Bird Ringing Centre, National Museum in Prague. The minimum age of each bird was  
149 estimated based on a combination of the ringing records and plumage age characteristics at their first  
150 capture (Svensson & Baker, 1992).

#### 151 **Plasma testosterone assay**

152 Plasma T was quantified in duplicates using a Testosterone ELISA Kit (480 solid well; product No  
153 582701; Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions,  
154 with measuring T range 3.9-500 pg/ml and sensitivity 6 pg/ml, using 20  $\mu\text{l}$  of the plasma sample  
155 diluted 5.5 $\times$  as input. The measurement repeatability in duplicates was  $r = 0.91$  ( $p < 0.001$ ) and intra-  
156 and inter-assay variation coefficients (CV) were 13.01 (N =102) and 24.01 % (N = 6), respectively  
157 (see SMMO 2 in SI for details).

#### 158 **Condition-related traits**

159 For the haematological analysis we followed the procedure reported previously by Bauerová et al.  
160 (2020). From blood smears stained with Wright-Giemsa Modified stain (product No. WG128, Sigma-  
161 Aldrich, St. Luis, MO, USA), we calculated the differential white blood cell count using a light  
162 microscope with 100 $\times$  objective magnification (Olympus Corporation, Tokyo, Japan, type CX-31),  
163 which served to estimate the H/L ratio (see SMMO 3 in SI for details).

164 To evaluate the individual nutritional status in the moulting period, we performed a ptilochronological  
165 analysis using the previously collected rectrix samples (Vinkler, Schnitzer, Munclinger, & Albrecht,  
166 2012). After scanning all rectrices (scanner Epson V30) and post-processing the images in Corel  
167 Photo-Paint X3 software (Corel Corporation, Ottawa, Canada), we estimated the FGR as the mean  
168 growth bar width in a segment of 10 growth bars with the centre located at 2/3 of the feather length  
169 using ImageJ software (v. 2.0.0; Schindelin et al. 2015; see SMMO 4 in SI for details).

170 From the scanned digital images of the black breast stripe ornaments, the breast stripe area was  
171 measured in Adobe PHOTOSHOP CS. 2 software (v. 10.0; Adobe Systems, San Jose, USA) according  
172 to Bauerová et al. (2017). For 20 randomly selected individuals, the repeatability of the measurement  
173 was found to be high ( $r = 0.9$ ,  $p < 0.001$ ).

174 Yellow carotenoid-based breast ornament was analysed using an Avaspec 2048 spectrometer with an  
175 Avalight XE light source and the Avasoft 7.0 processing system (Avantes, Eerbeek, Netherlands). For  
176 each individual, we measured the colour of a layer of 20-25 carotenoid-based feathers fixed on a glass  
177 slide (according to Quesada and Senar 2006). As estimates of the yellow colouration parameters, we  
178 calculated the chroma (the difference between the reflectance at 700 nm and 450 nm, relative to the  
179 reflectance at 700 nm; the interval for absorbance of carotenoid pigments is 450–700 nm) and total  
180 brightness (total reflectance, i.e. the sum of reflectance in visible light from 300 to 700 nm) from the  
181 spectral data (Montgomerie, 2006; see SMMO 5 in SI and Svobodová et al. 2018 and Albrecht et al.  
182 2009 for more details).

183

## 184 **Statistical analyses**

185 Statistical analyses were performed using R software (v. 3.4.1; R Core Team, 2017). First, to control  
186 for technical factors possibly affecting plasma T measurements (i.e. those that were not biologically  
187 relevant), we built a Linear Model (LM) with log-transformed T ( $T_{\text{raw}}$ ) as the dependent variable, and  
188 testosterone plate (batch in which the sample was measured), time of capture and handling time (i.e.  
189 the time between capture and blood sampling) as explanatory variables (model M1, Table S3 in SI).



190 We then used the residuals for testosterone from this model (termed M1) as ‘controlled T values’  
191 (further referred to as T) in all subsequent analyses.

192 We checked the inter-annual stability of T and condition-related traits separately for both sexes using  
193 two complementary calculations of repeatability, both based on variance components from Linear  
194 Mixed Models (LMMs) with individual ID as a random factor using the rptR package, version 9.22  
195 (Stoffel, Nakagawa, & Schielzeth, 2017). First, we assessed the inter-annual repeatability of absolute  
196 trait values (further referred to as absolute repeatability), and second, the inter-annual repeatability of  
197 the weighted relative rank of the trait in the population (i.e. relative order for a given trait weighted by  
198 the number of observations in each year; further referred to as individual rank consistency). Some  
199 traits can function as relative traits that possess low inter-annual repeatability in absolute values, but  
200 their relative status (rank) is maintained in a population between years (Senar & Quesada, 2006).

201 To see whether T levels undergo similar trajectories over the lifetime of both sexes, we tested for sex-  
202 specific age-related changes in plasma T levels in an LMM model (M2) with T levels used as a  
203 dependent variable, and sex, age (also as a quadratic term), their interactions (sex:age, sex:age<sup>2</sup>),  
204 Julian date (the date of capture converted to the Julian calendar), mean air temperature (mean air  
205 temperature seven days before catching; SMMO 1), tarsus length and selected condition-related traits  
206 (mass, FGR, H/L ratio and H/L ratio:sex) as fixed effects. The year of capture and individual ID were  
207 used as variables with random intercept effects, while the age was given a random slope effect within  
208 the individual ID (age|ID) to allow for inter-individual variations in age-related changes.

209 To investigate relationships between plasma T levels and ornament expression, we built LMM models  
210 separately for both sexes with the same random factors as in M2 and breast stripe area (M3-4), yellow  
211 chroma (M5-6) and yellow brightness (M7-8) used as dependent variables. In these models, the  
212 explanatory variables used as fixed effects were age, T, tarsus length, Julian date, and condition-  
213 related traits (mass, FGR, H/L ratio).

214 To achieve normality in model residuals (checked using the Shapiro-Wilk normality test), some  
215 response variables were log-transformed before model testing (Table S3 in SI). Minimum adequate

216 models (MAMs; defined here as models with all fixed terms significant at the level of  $p \leq 0.05$  or with  
217 marginally insignificant terms at the level of  $p < 0.10$ ) were selected by backward elimination of non-  
218 significant terms from the full models (Crawley, 2013). The backward elimination steps in the models  
219 were checked by changes of deviance with accompanied changes in degrees of freedom (ANOVA)  
220 and Akaike information criterion (AIC) using F statistics. For LMMs models, the packages nlme;  
221 version 3.1 (Pinheiro, Bates, DebRoy, & Sarkar, 2019) and lmer4; versions 1.1 (Bates, Mächler,  
222 Bolker, Benjamin, & Walker, Steven, 2015) were used.

## 223 **Results**

### 224 *Inter-annual stability and individual rank consistency in T levels*

225 In our set of 105 samples originating from 49 re-captured individuals, mean plasma  $T_{\text{raw}}$   
226 concentrations were  $393.93 \pm 388.24$  pg/ml in males ( $N = 28$ ) and  $110.43 \pm 74.78$  pg/ml in females ( $N$   
227  $= 21$ ; see Table S1 for plasma  $T_{\text{raw}}$  concentrations across the age cohorts). Given the differences in T  
228 levels between the sexes (unpaired two-sample t-test;  $t = 7.185$ ,  $df = 91.685$ ,  $p < 0.001$ ), we calculated  
229 their inter-annual T stability separately. The absolute inter-annual repeatability was lower for males ( $r$   
230  $= 0.103$ ;  $p > 0.05$ ) than for females ( $r = 0.576$ ;  $p < 0.001$ ) (Fig. 1A; Table S2A and Fig. S2A-B in SI),  
231 but not considerably different when relative ranks were taken into account (in males  $r = 0.411$ ;  $p =$   
232  $0.008$ ; in females  $r = 0.489$ ;  $p = 0.005$ ; Fig. 1B; Table S2B in SI). Absolute T levels of two-year-old  
233 individuals did not correspond well with their levels in subsequent years, because excluding  
234 observations of the two-year-old individuals from the data set considerably increased the absolute T  
235 inter-annual repeatability in males ( $r = 0.385$ ;  $p = 0.045$ ), but not in females ( $r = 0.59$ ;  $p = 0.016$ ;  
236 Table S2A).

### 237 *Age-dependent changes in plasma T levels*

238 Next, we tested whether T levels change during the lifetime (MAM2; Table 1, Table S3 in SI). We  
239 found a significant effect of age (age: slope = 0.032;  $p = 0.003$  and age<sup>2</sup>: slope = 0.462;  $p = 0.059$ ) on  
240 T levels with sex-specific interactions (age<sup>2</sup>:sex slope = -3.390,  $p = 0.032$  and age:sex slope = -2.887,

241  $p = 0.005$ ), with male T concentrations peaking in middle-age and decreases in older birds, while in  
242 females levels were stable throughout life (Fig. 2).

### 243 *Inter-annual stability of condition-related traits and their relationship with T levels*

244 From all condition-dependent traits, only the breast stripe area (Fig. S3A-B in SI), yellow chroma  
245 (Fig. S4A-B in SI), body mass (Fig. S8A-B in SI) and the H/L ratio in females (Fig. S6B in SI) were  
246 somewhat inter-annually repeatable in absolute values, with differences between sexes and young and  
247 adult individuals (Fig. 1; Table S2A and S2B in SI). In contrast, brightness of the yellow carotenoid  
248 ornaments (Fig. S5A-B in SI), H/L ratio in males (Fig. S7A in SI), and FGR (Fig. S6A-B in SI) had no  
249 detectable repeatability. This pattern of relatively compromised absolute repeatability was in most  
250 cases in contrast to the reasonably high-rank consistency (Fig. 1 and Table S2B in SI).

251 Finally, we investigated whether T levels predict FGR, body mass, and the extent of ornamental trait  
252 expression. Neither FGR nor body mass were associated with T levels (MAM2; Table 1, Table S3 in  
253 SI). Also, we found no associations between the breast stripe area and T levels in either sex (MAM3  
254 and MAM4, Table 1, Table S3 in SI), and no relationships for the carotenoid- ornament chroma  
255 (MAM5 and MAM6 in Table 1, Table S3 in SI). However, unlike in females (MAM8, Table 1, Table  
256 S3 in SI), our results showed a strong positive relationship between T levels and yellow plumage  
257 brightness in males (slope = 5834.87,  $p = 0.001$ , MAM7 in Table 1, Table S3 in SI and Fig. 3).

### 258 **Discussion**

259 In this study, we found for the first time that plasma T levels in birds are inter-annually stable in both  
260 sexes, yet with a higher degree of stability in females. Male T levels showed a quadratic dependence  
261 on age with a midlife peak. In contrast to males, female T levels were generally stable throughout their  
262 lifetime. We did not find any relationships between T levels and most condition-related traits tested,  
263 although most of these traits were also inter-annually rank-stable. The only significant and strong  
264 positive association was found between male T levels and brightness of the yellow carotenoid-based  
265 plumage ornaments, presumably serving as an honest signal in sexual selection.

266 Given the demanding sampling design of longitudinal studies, inter-annual plasma T level stability had  
267 not been properly tested in birds. This study provides the first evidence that avian plasma T levels are  
268 inter-annually stable in both sexes. The relatively surprising finding of higher inter-annual T stability  
269 in females compared to males appears to result from ontogenetic fluctuations in male T levels. This is  
270 supported by the finding that male T repeatability increased when the observations of young (two-  
271 year-old) individuals were excluded, indicating higher T stability after reaching reproductive maturity.  
272 However, the lower differences between sexes in individual T consistency ranks indicate that males  
273 maintained their relative ranks in the population rather than absolute plasma T levels. Similarly, T  
274 levels have been found to be inter-annually stable in the plasma of human women (Burger et al.,  
275 2000) and beluga whales (Montano, Robeck, Steinman, & O'Brien, 2017) and lesser mouse lemur  
276 (*Microcebus murinus*) males (Perret, 1992). Furthermore, also consistent with our results, higher T  
277 levels in ontogeny appear to be linked with lower T stability, as seen in a dataset of pubertal boys  
278 (Klipker, Wrzus, Rauers, Boker, & Riediger, 2017). In males, steep increases in T levels towards peak  
279 levels followed by steep decreases impair repeatability in the data, in contrast to females with  
280 relatively flat T trajectories.

281 In great tit males, we observed a polynomic dependence of T levels on age, with an initial three-fold  
282 increase, a peak in middle-aged individuals (minimum estimated age of four years), and a steadily  
283 decreasing trend later in life. In birds, the production of various hormones has been suggested to  
284 decline with age, co-occurring with a decrease in reproductive output (Ottinger, 1996). However, our  
285 results in males are well consistent with longitudinal data in humans (Vermeulen et al. 1972; Harman  
286 et al. 2001), as well as in long-lived Florida scrub-jays (*Aphelocoma coerulescens*), with plasma T  
287 levels peaking in middle-age (in jays there was also a ca. three-fold initial increase in T levels  
288 followed by a decrease towards the minimum T levels in the oldest, yet fertile birds; Wilcoxon et al.  
289 2013). Although no other current avian study has adopted a longitudinal design that prevents the risk  
290 of biased interpretations due to selective disappearance in different age cohorts, further support for our  
291 results can be found in some cohort studies. In short-lived laboratory-kept Japanese quails (*Coturnix*  
292 *japonica*; Balthazart et al. 1984), plasma T levels dropped almost two times in the oldest age class

293 compared to younger ones. Similarly, in longed-lived common terns (*Sterna hirundo*), male plasma T  
294 levels first sharply increased and reached a peak at maturity (2-5 years), then started to moderately  
295 decrease with age (Nisbet et al., 1999). An analogous trend suggesting the effects of hormonal  
296 senescence was also described for feather T levels in barn swallows (Adámková et al. 2019). These  
297 results are in contrast with most other avian studies (none using a longitudinal design), which have not  
298 detected any age-related changes in male T levels. The lack of such a relationship in studies comparing  
299 young vs adult individuals (e.g. in the house finch, *Haemorhous mexicanus*, Belthoff et al. 1994; the  
300 Florida scrub-jay *Aphelocoma coerulescens*, Schoech et al. 1996 and the magnificent frigatebird,  
301 *Fregata magnificens*, Madsen et al. 2007) may have been caused, for example, by the delayed  
302 breeding of younger birds at the time of their capture. Other studies with no detected relationship  
303 between age and T using cross-sectional designs (e.g. in the cliff swallow; *Petrochelidon pyrrhonota*;  
304 Smith et al. 2005), and the superb fairy-wren; *Malurus cyaneus*; Peters et al. 2002) may have been  
305 biased by selective disappearance (Zhang, Vedder, Becker, & Bouwhuis, 2015). Thus, despite  
306 previous controversies arising from cohort-based studies, our data support the universal male  
307 ontogenetic patterns in T levels across birds and mammals.

308 While physiologically important (Goymann & Wingfield, 2014), no longitudinal study has yet  
309 examined whether female T undergoes age-related changes through ontogeny in birds. Recently,  
310 female T levels have been shown to decline with age in two passerines, the pied flycatcher (*Ficedula*  
311 *hypoleuca*; Moreno et al. 2014) and the barn swallow (Adámková et al., 2019) both using cross-  
312 sectional data. In contrast, in our study female plasma T levels remained stable during their lifetime.  
313 Other longitudinal studies are needed to resolve whether there are ontogenetic parallels between  
314 mammals and birds in female T levels.

315 Unlike yellow plumage brightness, FGR and male H/L, the body mass and female H/L as well as the  
316 black breast stripe area and yellow plumage chroma were shown to be repeatable. This likely partially  
317 corresponds with stronger genetic components, especially in melanin-based ornaments (Grunst,  
318 Rotenberry, & Grunst, 2014; Senar & Quesada, 2006). The absence of repeatability in brightness may

319 reflect its higher phenotypic plasticity with strong seasonal effects (Senar, Figuerola, & Pascual, 2002)  
320 but see Senar and Quesada, (2006).

321 Elevated T levels induce higher aggressivity, mating, and explanatory behaviours with more social  
322 interactions, all of which may interfere with body condition and increase physiological stress (Braude,  
323 Tang-Martinez, & Taylor, 1999) for the benefit of increased fitness, and reproduction-related traits  
324 including ornaments. Despite links between T levels and several condition-dependent traits being  
325 widely described in other studies (for evidence under the Immunocompetence hypothesis; Folstad and  
326 Karter 1992 or the Oxidation handicap hypothesis; Alonso-Alvarez et al. 2008, see e.g. Evans et al.  
327 2000; Galván and Alonso-Alvarez 2010; Duckworth et al. 2004), in our study, we found only limited  
328 evidence supporting these associations. We did not observe the predicted negative link between T and  
329 body condition or the positive relationship between T levels and the H/L ratio, an indicator of  
330 physiological stress. This is, however, consistent with several other studies in passerine birds  
331 (Buchanan et al. 2003; Seddon and Klukowski 2012).

332 Similarly, little support was found for links between T and plumage ornaments. This may result from  
333 the fact that the effects of T on bird coloration are often complex and condition-dependent, and can  
334 therefore adopt variable directions or remain entirely lacking (Fargallo et al. 2007; Alonso-Alvarez et  
335 al. 2009; Rull et al. 2016). Furthermore, plasma T levels influence pigment deposition into ornaments  
336 only during the moulting period in the previous season (Kimball, 2006), so the absence of these  
337 relationships may also result from low individual inter-seasonal T rank stability, which remains  
338 unknown (Kempnaers et al. 2008; but see Adámková et al. 2019). However, we observed a strong  
339 positive association between T plasma levels and the brightness of the yellow plumage ornament in re-  
340 captured males. We hypothesise that this relationship may reflect the production of preen oils that are  
341 spread on feathers as protection against water and parasites (Moreno-Rueda, 2017). Uropygial gland  
342 preen oil secretion has been shown to positively correlate with plasma T levels (Amet, Abalain,  
343 Daniel, Di Stefano, & Floch, 1986; Floch, Floch, Morfin, & Daniel, 1988), which can influence the  
344 spectral structural properties of feathers (Møller & Mateos-González, 2018). Importantly, higher  
345 brightness indicates individual quality in tits (Senar et al., 2002).

346 Our findings showing that avian plasma T levels are inter-annually stable in both sexes provide an  
347 important basis for further research linking hormonal levels with other physiological and life-history  
348 traits. The individual rank consistency validates the use of temporarily-variable T levels to explain  
349 hormonal effects in traits measured at different time points during the lifetime, which is particularly  
350 important when investigating condition-related traits in cross-cohort datasets. Nevertheless, consistent  
351 with mammals our results also demonstrated age-dependent changes in T levels in males, indicating  
352 that this pattern may be universal across vertebrates. Since the inter-annual stability in condition-  
353 related traits mostly lacked any clear relationship to plasma T concentrations in our study, we suggest  
354 that a detailed description of the regulatory effects of T in correlative studies may require more  
355 detailed information on the individual histories of investigated individuals. This research opens the  
356 way to further understanding of context-dependency and variation in the hormonal regulation of  
357 fitness-related trait expression.

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372

373 **Authors' contributions**

374 M.T., M.V., and T.A. designed the study. M.T., T.K., H.V., J.S., H.P., P.B., J.K., T.A., and M.V.  
375 collected samples. M.T., T.K., and J.E. performed the laboratory analysis. M.T. and M.V. interpreted  
376 the data and drafted the manuscript. All authors contributed with their comments to the final approved  
377 version of the manuscript.

378

379 **Data availability statement**

380 The data supporting the results are archived in the Dryad repository:

381 <https://datadryad.org/stash/share/dqno1iviZi4v00C3nfBWIMwZUWkVsl3rzCaYrn3JgCc>

382



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600

601 **Tables and figures**

602 **Table 1** Minimum adequate models (MAMs) for re-captured great tits. For Linear Mixed Models (MAM2 – 8), the year of capture and individual ID were  
603 used as random effects with random intercepts. Age was given as a random slope effect within the individual ID (age|ID). Residuals for testosterone (T) from  
604 full Linear Model 1 were used in all other models. Slope  $\pm$  SE values are provided only for continuous variables. *P*-values at statistical significance level  $p \leq$   
605 0.05 are in bold. T – plasma testosterone concentration [pg/ml], breast stripe – the black breast stripe area [mm<sup>3</sup>], individual – individual identification based  
606 on bird ringing, sex, age – minimal estimated age based on bird ringing (age<sup>2</sup> – polynomial term), T\_ave7 – mean daily temperature 7 days before catching,  
607 FGR – feather growth rate, Jul.date – Julian date from the beginning of the year, mass – body mass as the ratio of weight and tarsus length, HL – HL ratio  
608 (number of heterophils/ number of lymphocytes), brightness – brightness of yellow carotenoid ornament, yellow chroma – chroma of the yellow carotenoid  
609 ornament, N<sub>obs</sub> – number of observations, N<sub>ind</sub> – number of individuals

610

611

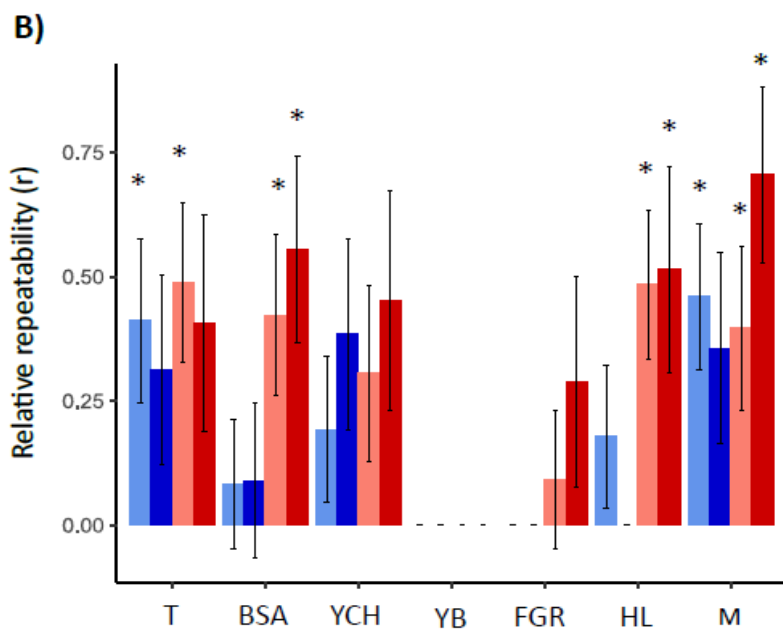
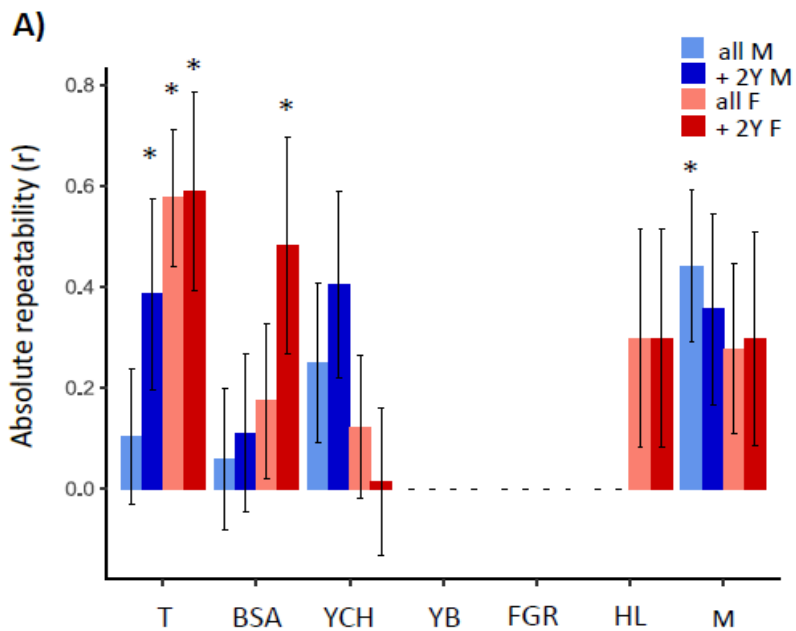
Minimum adequate model	Slope ± SE	F	Df	p	N <sub>obs</sub> /N <sub>ind</sub>
MAM 1 $\log(T_{raw}) \sim T_{plate}$		2.766	5/104	0.022	105/49
MAM 2 $T \sim \text{sex} + \text{age} + \text{age}^2 + T_{ave7} + \text{sex}:\text{age}^2 + \text{sex}:\text{age} + (1 \text{year}) + (\text{age} \text{individual})$		43.005	6/6	<0.001	105/49
sex	0.990 ± 0.172	31.408	3/9	<0.001	
age	0.032 ± 0.100	15.870	4/8	0.003	
age <sup>2</sup>	0.462 ± 1.029	5.665	2/10	0.059	
T <sub>ave7</sub>	-0.056 ± 0.031	3.248	1/11	0.071	
sex:age <sup>2</sup>	-3.390 ± 1.633	4.589	1/11	0.032	
sex:age	2.877 ± 1.690	10.459	2/10	0.005	
MAM 3 (males): $\log(\text{breast stripe}) \sim \text{FGR} + (1 \text{year}) + (\text{age} \text{individual})$	165.72 ± 60.18	7.120	1/7	0.008	58/28
MAM 4 (females): $\text{breast stripe} \sim \text{FGR} + \text{Jul.date} + (1 \text{year}) + (\text{age} \text{individual})$		12.743	2/6	0.002	47/21
FGR	0.278 ± 0.144	3.414	1/7	0.065	
Jul.date	-0.013 ± 0.003	12.309	1/7	<0.001	
MAM 5 (males): $\text{yellow chroma} \sim \text{HL} + (1 \text{year}) + (\text{age} \text{individual})$	0.001 ± 0.001	2.004	1/6	0.157	58/28
MAM 6 (females): $\text{yellow chroma} \sim \text{HL} + \text{mass} + (1 \text{year}) + (\text{age} \text{individual})$		6.687	2/6	0.035	46/21
HL	0.001 ± 0.001	3.757	1/7	0.053	
mass	0.003 ± 0.002	2.821	1/7	0.093	
MAM 7 (males): $\text{brightness} \sim \text{age} + \text{age}^2 + \text{mass} + \text{HL} + \text{Jul.date} + \text{FGR} + \text{T} + \text{T:Jul.date} + (1 \text{year}) + (\text{age} \text{individual})$		37.744	8/6	<0.001	58/28
age	792.570 ± 245.120	18.672	2/12	<0.001	
age <sup>2</sup>	-4631.380 ± 1387.940	9.485	1/13	0.002	
mass	-21340.220 ± 5590.590	14.130	1/13	<0.001	
HL	-427.940 ± 166.220	7.077	1/13	0.008	
Jul.date	32.220 ± 16.070	8.397	2/12	0.015	
FGR	-1570.390 ± 910.940	3.067	1/13	0.080	
T	5834.870 ± 1819.610	13.524	2/12	0.001	
T:Jul.date	-40.240 ± 13.660	8.311	1/13	0.004	
MAM 8 (females): $\text{brightness} \sim \text{age} + (1 \text{year}) + (\text{age} \text{individual})$	449.800 ± 208.000	4.879	1/6	0.027	47/21

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613 **Figures**

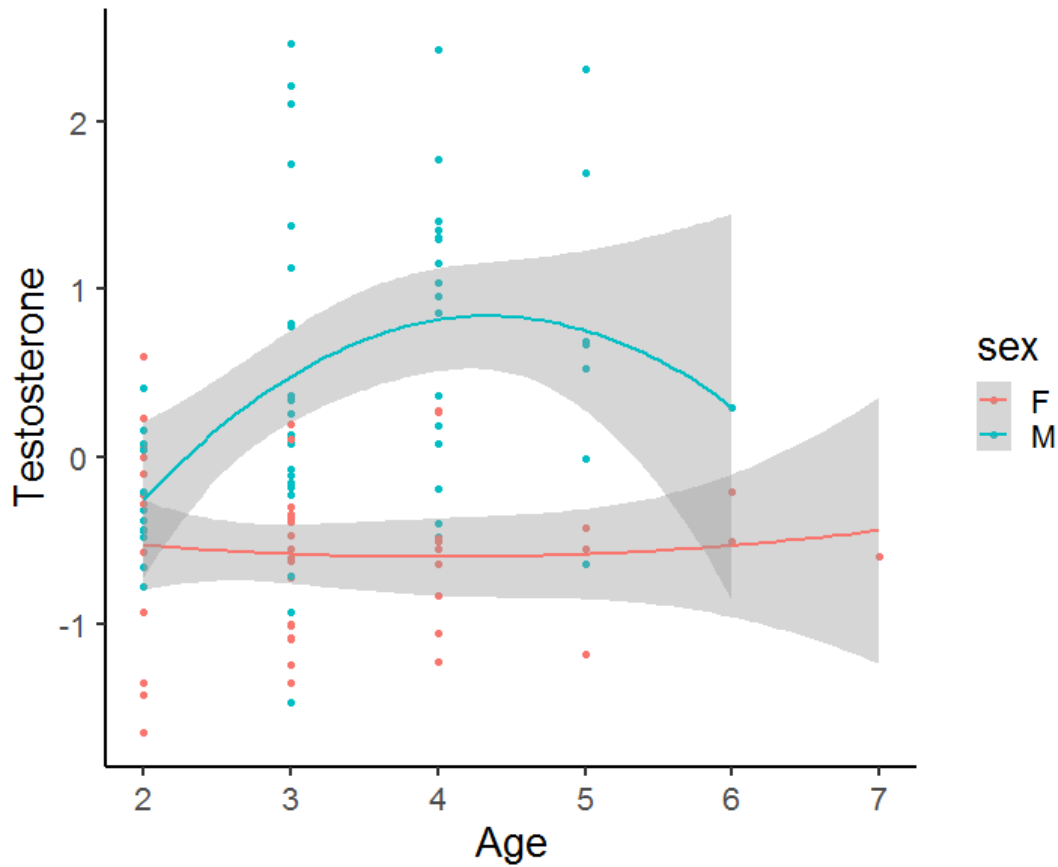
614 **Figure 1** Inter-annual individual absolute (A) and relative (B; also termed individual rank consistency)  
615 repeatability of testosterone and condition-related traits in repeatedly captured great tits. The  
616 repeatability is indicated by the bar height with the standard error, separately for each category (for  
617 cases with no repeatability no bars are shown). *P*-values at statistical significance level  $p \leq 0.05$  are  
618 marked with an asterisk (see Table S2 in SI for details). T – residuals for plasma testosterone, BSA –  
619 the black breast stripe area, YCH – chroma of yellow ornamental plumage, YB – brightness of yellow  
620 ornamental plumage, FGR – feather growth rate, i.e. daily growth bar width increase in rectrices, HL –  
621 the ratio of heterophils to lymphocytes in peripheral blood, M – body mass (weight relative to tarsus  
622 length). The calculations were done separately for both sexes and with either all observations or after  
623 excluding observations of young birds (two-year-olds; 2Y): All M – all male individuals ( $N_{\text{ind}} = 28$ ,  
624  $N_{\text{obs}} = 58$ ), +2Y M – only male individuals older than two years ( $N_{\text{ind}} = 17$ ,  $N_{\text{obs}} = 36$ ), all F – all  
625 female individuals ( $N_{\text{ind}} = 21$ ,  $N_{\text{obs}} = 47$ ), +2Y F – only female individuals older than two years ( $N_{\text{ind}} =$   
626  $11$ ,  $N_{\text{obs}} = 24$ )

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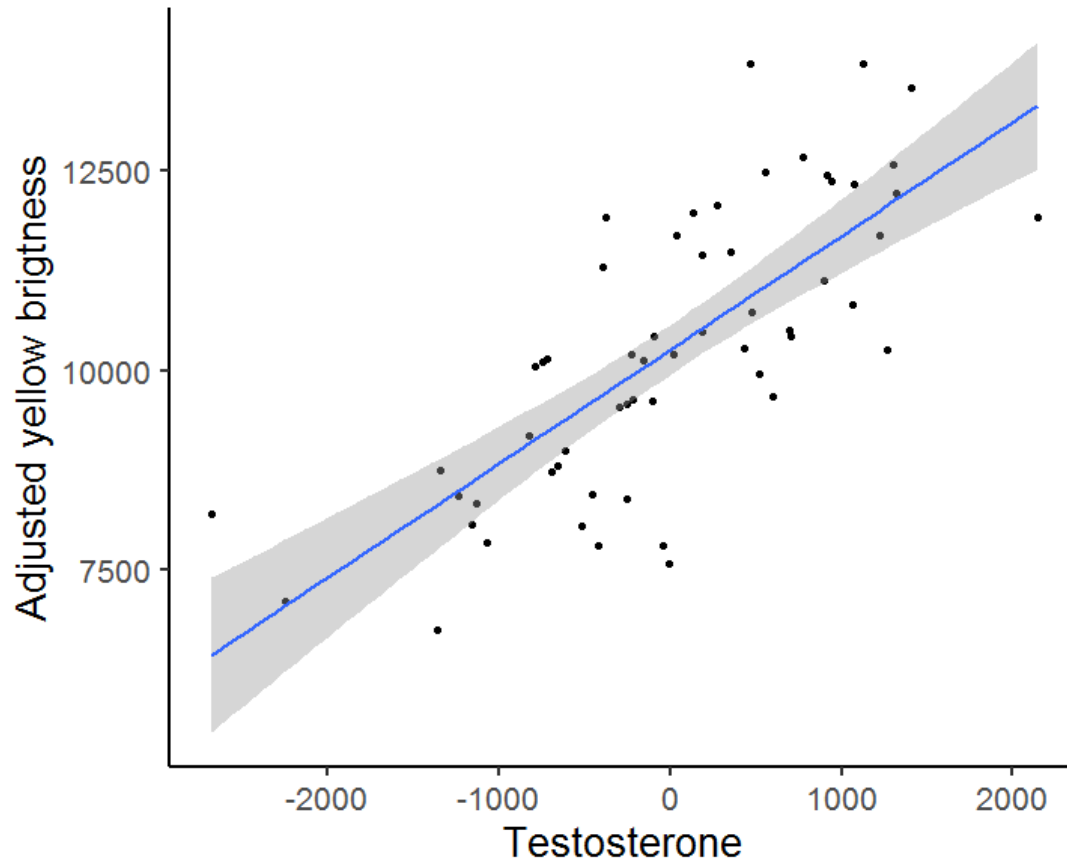
628

629 **Figure 2** The relationship between plasma testosterone (T) levels and age in re-captured great tit males  
630 and females ( $N_{ind} = 49$ ,  $N_{obs} = 105$ ). Plasma T levels are shown from minimal adequate model 2 (Table  
631 1), with confidence intervals indicated by grey shaded areas. Age is the minimal estimated age based  
632 on bird ringing



633

634 **Figure 3** The relationship between brightness and testosterone (T) levels in repeatedly captured males  
635 ( $N_{\text{ind}} = 28$ ,  $N_{\text{obs}} = 58$ ). The yellow brightness is shown as residuals from minimal adequate model 7  
636 (Table 1), with the confidence interval indicated by the grey shaded area



637