

1 **Title: Effects of experimentally sustained elevated testosterone on incubation behaviour and**
2 **reproductive success in female great tits (*Parus major*)**

3 **Authors:** Berber de Jong^{a,b,c}, Luc Lens^b, Seyed Mehdi Amininasab^{c,d}, Kees van Oers^e, Veerle M. Darras^f,
4 Marcel Eens^g, Rianne Pinxten^{g,h}, Jan Komdeur^c & Ton G.G. Groothuis^a

5 **Affiliations:**

6 a. Behavioural Biology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, P.O.
7 Box 11103, 9700 CC, Groningen, The Netherlands

8 b. Terrestrial Ecology Unit, Department Biology, Ghent University, BE-9000, Ghent, Belgium.

9 c. Behavioural and Physiological Ecology, Groningen Institute for ELIFES, University of Groningen, P.O.
10 Box 11103, 9700 CC, Groningen, The Netherlands

11 d. Department of Environmental Science, Behbahan Khatam Alanbia University of Technology, Iran

12 e. Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 50, 6700 AB,
13 Wageningen, The Netherlands

14 f. Laboratory of Comparative Endocrinology, K.U. Leuven, Naamsestraat 61, B-3000 Leuven, Belgium

15 g. Behavioural Ecology & Ecophysiology Research Group, Department of Biology, University of Antwerp,
16 Universiteitsplein 1, B-2610 Wilrijk, Belgium

17 h. Didactica Research Group, Faculty of Social Sciences, University of Antwerp, B-2000, Antwerp,
18 Belgium

19 **Corresponding author:**

20 Berber de Jong

21 Behavioural and Physiological Ecology

22 Groningen Institute for Evolutionary Life Sciences

23 University of Groningen,

24 P.O. Box 11103, 9700 CC,

25 Groningen, The Netherlands

26 Tel: +31-629478683

27 Email: berber.dejong@gmail.com

28

29 **ABSTRACT**

30 In many seasonally breeding birds, female and male testosterone (T) levels peak at the start of the
31 breeding season, coinciding with pair bonding and nesting activities. Shortly after the onset of egg laying,
32 T levels slowly decline to baseline levels in both sexes, but more rapidly so in females. During this period,
33 T in males may still function to facilitate territorial behaviour, mate guarding and extra pair copulations,
34 either via short lasting peaks or elevated basal levels of the hormone. In some species, however, males
35 become insensitive to increased T after the onset of egg laying. It has been postulated that in these
36 species bi-parental care is essential for offspring survival, as T is known to inhibit paternal care. However,
37 only very few studies have analysed this for females. As females are heavily involved in parental care,
38 they too might become insensitive to T after egg laying. Alternatively, because territorial defence, mate
39 guarding and extra pair copulations are expected to be less important for females than for males, they
40 may not have had the need to evolve a mechanism to become insensitive to T during the period of
41 maternal care, because their natural T levels are never elevated during this part of the breeding season
42 anyway. We tested these alternative hypotheses in female great tits (*Parus major*). Male great tits have
43 previously been shown to be insensitive to T after egg laying with regard to nestling feeding behaviour
44 (but not song rate). When females had started nest building, we experimentally elevated their T levels up
45 to the nestling feeding phase, and measured incubation behaviour (only females incubate) and
46 reproductive success. T did not significantly affect nest building or egg laying behaviour, although egg
47 laying tended to be delayed in T females. Females with experimentally enhanced T maintained lower
48 temperature during incubation but did not spend less time incubating. This might explain the reduced
49 hatching success of their eggs, smaller brood size and lower number of fledglings we found in this study.
50 As in this species T-dependent behaviour by females during the phase of parental care is not needed, the
51 results support the hypothesis that in this species the need for selection in favour of T-insensitivity did
52 not occur.

53 **Keywords:** *Parus major*; experimentally elevated testosterone; incubation behaviour; reproductive
54 success; essential mating effort hypothesis

55

56 1. INTRODUCTION

57 Testosterone (T) concentrations in males remain important for reproductive success in many seasonally
58 breeding birds after territory establishment and pairing, although they decline gradually after the onset
59 of egg laying (Ketterson et al., 2005; Wingfield, 1990). T is known to facilitate mate guarding, extra-pair
60 fertilization, secondary female acquisition, and/or territorial defence during parental care. For example,
61 experimentally elevated T has been shown to increase singing behaviour to attract additional mates
62 (European starling (*Sturnus vulgaris*), De Ridder et al., 2000), courtship behaviour (house sparrow (*Passer*
63 *domesticus*), Hegner and Wingfield, 1987; red bishop (*Euplectes orix*), Edler et al., 2011), extra-pair
64 fertilization rate (dark-eyed junco (*Junco hyemalis*), Raouf et al., 1997) and attractiveness to females
65 (dark-eyed junco, Enstrom et al., 1997) later on in the breeding season. Yet T has also been shown to
66 suppress incubation behaviour (spotted sandpiper (*Actitis meularia*), Oring et al., 1989; yellow-legged
67 gull (*Larus cachinnans*), Alonso-Alvarez, 2001) and nestling provisioning (Hegner and Wingfield, 1987) in
68 males. Thus, elevated levels of T in males appear to moderate the trade-off between mating effort and
69 parental effort (Adkins-Regan, 2005). In certain species, however, males do not respond to T elevation
70 after egg laying with a reduction of parental behaviour (lapland longspur (*Calcarius lapponicus*), Hunt et
71 al., 1997; chestnut-collared longspur (*Calcarius ornatus*), Lynn et al., 2002; great tit (*Parus major*), Van
72 Duyse et al., 2002; black-tailed gull (*Larus crassirostris*), Kazama et al., 2011). Such variation in sensitivity
73 of male parental behaviour to T after egg laying may be explained by the essential paternal care
74 hypothesis, which postulates that in species where bi-parental care is essential for the survival of
75 offspring, males, becomes insensitive to T (in terms of their parental behaviours) during the period of
76 increased paternal care (Lynn et al., 2002; Lynn, 2008) in order to avoid the detrimental effects of the
77 hormone.

78 In many avian species, female testosterone (T) levels rise, as in males, at the start of their breeding
79 season but decline shortly after the start of egg laying (Ketterson et al., 2005). Although less well studied
80 than in males, there is some evidence that the seasonal peak in T levels might be beneficial for females
81 too. For example, early peak T levels are linked to female aggression (red-winged blackbird (*Agelaius*
82 *phoeniceus*), Searcy, 1988; European starling, Sandell, 2007; blue tits (*Cyanistes caeruleus*), de Jong,
83 2013; tree swallow (*Tachycineta bicolor*), Rosvall, 2013a, b). This can help securing male care by
84 outcompeting rivaling females (great tit, Slagsvold 1993, dunnock (*Prunella modularis*), Langmore et al.,
85 2002; Sandell, 2007). Also, more aggressive females had higher reproductive success (dark-eyed junco,
86 Cain and Ketterson, 2012). Females of many species also remain sensitive to elevated T levels later in the
87 breeding season. However, in contrast to males, this prolonged sensitivity to T in females has mainly
88 been associated with costs that could reduce reproductive success. For example, experimentally
89 prolonged elevated T levels have been shown to delay the onset of egg laying (Searcy, 1988; dark-eyed
90 junco, Clotfelter et al., 2004; zebra finch (*Teaniopygia guttata*), Rutkowska et al., 2005), decrease
91 incubation temperature (Rosvall, 2013a,b), reduce brooding of nestlings (dark-eyed junco, O'Neal et al.,
92 2008), and decrease the number of hatchlings and fledglings (spotless starling (*Sturnus unicolor*), Veiga
93 and Polo, 2008; spotless starling, Lopez-Rull and Gil, 2009; Rosvall, 2013a,b; dark-eyed junco, Gerlach
94 and Ketterson, 2013) in various passerine birds. In other passerine species, however, prolonged
95 experimentally elevated T levels does not affect the onset of egg laying (de Jong, 2013) or incubation

96 behaviour (European starling, Sandell et al., unpublished manuscript cited in Ketterson et al., 2005).
97 Moreover, a few studies suggest that remaining sensitive to elevated T levels after egg laying might be
98 advantageous for females. For example, prolonged elevated T levels are linked to female aggression
99 (Searcy, 1988; Sandell, 2007; Rosvall, 2013a,b) which can secure male care by enabling females to
100 outcompete rivals (European starling, Sandell, 1998; Langmore et al., 2002). A similar suggestion was
101 made by Rosvall 2013a, demonstrating sensitivity to T during the period of parental care in tree swallow.

102
103 Thus, in several bird species, female parental care appears to remain sensitive to elevated T levels after
104 egg laying. This has been explained by the “essential mating effort hypothesis”, (Rosvall, 2013a, b). This
105 hypothesis postulates that females in these species did not evolve insensitivity to T, since sensitivity to T
106 is required for mediating other behaviours that are important for reproductive success, such as securing
107 mates and nest sites, also during the period of parental care. Therefore, the benefits remaining sensitive
108 to T by T dependent mating effort may outweigh the potential costs of suppression of parental care.

109 However, the majority of studies of sensitivity to T in females after egg laying have applied T
110 implantations to test behavioural sensitivity. This may have induced much higher T levels than the
111 endogenously produced low levels during that time period. It is therefore conceivable that females react
112 to these artificially elevated levels, showing sensitivity to the hormone, because selection for becoming
113 insensitive to the hormone was never necessary. In such species the costs of maintaining T production
114 and remaining sensitive to it (reduction of parental care) would outweigh the benefits. This may be the
115 case in many species in which females during the phase of parental care do not participate in nest
116 defence or mate competition. On the other hand, aggression, sexual behaviour and parental care are
117 influenced by partly the same brain areas, all containing androgen receptors (Rosvall 2013b). Therefore it
118 is also possible that females only become insensitive to T in their parental behaviour, but not in
119 aggressive and sexual behaviour. This is suggested by data on male great tits where experimentally
120 elevated T levels increased the expression of song while not affecting parental care (Van Duyse et al.,
121 2000).

122 In this study we examine the effects of sustained experimentally elevated T levels on incubation
123 behaviour and reproductive success in female great tits (*Parus major*). The great tit is a socially
124 monogamous species with bi-parental care, which is essential for the survival of the offspring (Bjorklund
125 and Westman, 1986a). Among females there is competition for males that own a territory (Gosler, 1993),
126 which might explain female-female aggression at the beginning of the breeding season (Slagvold, 1993).
127 Only females build nest and incubate eggs, but both parents provide food to their nestlings. The great tit
128 is one of the few species in which elevated T levels in males do not suppress paternal care measured as
129 food provisioning rate, although it does increase song rate (Van Duyse et al., 2000).

130 It is currently unclear whether female great tits remain sensitive to elevated T levels later in their
131 breeding season after egg-laying, when natural T levels have declined. By comparing reproductive
132 behaviour and reproductive success between females treated at the start of nest building with long
133 lasting T-implants or with empty implants (controls), we tested to what extent great tit females remain
134 sensitive to T. Since the effectiveness and pattern of incubation might be influenced by T and affect

135 hatching success, we also measured nest temperature during incubation. The only other similar study
136 that looked at experimentally elevated T levels and incubation behaviour in females was conducted on
137 tree swallows, a species in which females need to defend mates and their nest cavities against intruders
138 also during the phase of parental care (Rosvall 2013a). In great tits there is competition for nest cavities
139 during the winter / pre-breeding period (Gosler, 1993) but later in the season, when birds are incubating,
140 female-female aggression in the neighbourhood of the nest box has rarely been observed (more than 20
141 hours of personal observations BdJ). Also polygyny rarely occurs in this socially monogamous species
142 (Björklund and Westman, 1986b). This suggests that T levels during the parental care period are not
143 beneficial. During this period natural T levels are indeed low (Rost, 1990). Therefore, we hypothesised
144 that selection against remaining sensitive to T has never been needed and that females will still respond
145 behaviourally to artificially increased levels of this hormone. Under natural conditions female great tit
146 probably never experienced detrimental behaviours induced by elevated T levels later in the breeding
147 season. Therefore, it is unlikely that selection has acted on reducing sensitivity to T during this period. By
148 experimentally elevating T level for a long period we expect to expose these behaviours that might be
149 negatively affected by selection.

150

151 **2. MATERIALS & METHODS**

152 **2.1 Study area and study species**

153 The study was conducted in a population of great tits using nest boxes near the city of Antwerp, Belgium
154 (51° 10'N, 4° 17'E), during the spring of 2009. The study area consists of a park area with deciduous
155 forest containing 58 nest-boxes. Great tits in this population produced an average clutch of nine eggs (SE
156 ± 0.44) per year. Second clutches rarely occur in this population (personal observation BdJ). Full day
157 incubation starts after clutch completion and lasts on average thirteen days, although great tits already
158 incubate their eggs for short periods at night before clutch completion (Gosler, 1993). From early March
159 onwards, nest boxes were checked every other day to determine the onset of nest building. As soon as
160 females started nest building, they were captured at night (in nest boxes) or during the day (in food-
161 baited potter traps). At capture, all individuals were sexed (following Svensson, 1984) and banded with a
162 unique metal ring and three colour rings for individual identification. Age (second calendar year or older)
163 was determined based on the colour of the wing feather (following Cramp and Perrins, 1993).

164 When females were captured for implantation (see below), nests were checked daily to determine the
165 extent of nest building (see paragraph below), the onset of egg laying and incubation, and the clutch size.
166 The onset of incubation was determined when a female was found incubating her eggs or when the eggs
167 were found uncovered and warm. After females started incubating, nests were checked every second
168 day, while two days before expected hatching date, daily checks were resumed to determine hatching
169 date and brood size. The number of hatchlings and brood size were determined on day 6. Nestlings that
170 had died before day 6 were included in the number of hatchlings. When nestlings were 10 days old they
171 were ringed with a metal ring and their body mass was measured to the nearest 0.1g using a digital
172 balance. When nestlings were 15 days old, their body mass was measured again and their tarsus length
173 was measured to the nearest 0.1mm using a calliper. Near the end of the nestling phase (when nestlings

174 were ca. 17 days of age), daily nest checks were resumed to determine the fledging date and number of
175 fledged young.

176 **2.2 Implantation procedure**

177 Females were assigned randomly to a control (C) or a testosterone (T) treatment group, while nest
178 building stage taken into account, ensuring an approximately equal number of females with the same
179 nest building stage per treatment. Four nest building stages were distinguished: small parts of nesting
180 material present (stage 1), a solid layer of nesting material present (stage 2), a nest cup present but not
181 yet lined with hairs and feathers (stage 3), and a completed nest lined with hair and feathers (stage 4).
182 When a nest box was empty it was labelled as stage 0. If the nest stage was intermediate of two stages
183 the stage was assigned a half (e.g. a nest box had almost a solid layer but the bottom of the box was still
184 visible it would be called stage 1.5). A total of nine C birds and 12 T birds were implanted, of which a
185 total of seven C and seven T birds started breeding in our experiment (details see below). The average
186 nest stage at the time of implantation (2; 1–2.5) of all the implanted birds was not significantly different
187 between the treatments (mean nest building stage C females: 2; 1-2; mean nest building stage T females:
188 2; 0.5-2; Mann-Whitney U-test (1) = 48.5, Z = 0.39, P = 0.70), nor was it significantly different for the
189 birds that actually started breeding in the population (nest building stage for birds breeding in the
190 population: 1.75; 1-4; nest building stage for birds not breeding in the population: 2; 0-2; Mann-Whitney
191 U-test (1) = 22.5, Z = 0.26, P = 0.80). Just prior to implantation, females were weighed and their tarsus
192 length and third primary feather (P3) were measured to the nearest 0.1 mm with a stop-ruler. There was
193 no difference in body mass, tarsus length or P3 length between control and T groups prior to
194 implantation (all $P > 0.48$). All females of which the age was determined (C = 8; T = 9) were scored as
195 second calendar year birds.

196 Both T and C females were implanted with a 6-mm long silastic tube (Degania silicone; inner diameter
197 0.762 mm, outer Diameter 1.651 mm), which was sealed at both ends with silcon glue (Dow corning).
198 The implant was inserted subcutaneously along the left flank under local anaesthesia (Xylocaine, 10%
199 spray). After implantation the small incision was sutured with tissue glue (1 × 0.5 ml Histoacryl, Braun,
200 Germany). In T females, the implants were filled with 0.6 ± 0.015 mg crystalline testosterone (Fluka) over
201 a length of 2 mm. C females received empty implants. The amount of T used in our experiment was
202 determined based on a pilot study in which a higher dosage ($0.8\text{mg} \pm 0.025\text{mg}$) suppressed normal
203 breeding activities, such as egg laying and incubation behaviour, so the dosage used in our experiment
204 was slightly lower. All females were implanted between 16th of March and the 12th of April 2009, and
205 there was no difference in implantation date between the two treatments (independent t-test $t_{19} = -$
206 1.00 , $P = 0.33$), nor was there a difference in implantation date for those birds that started breeding in
207 the population (Mann-Whitney U-test (1) = 24.5, Z = 0.0, P = 1.0). Two days after implantation, one C
208 female was found dead. Additionally one C female and five T females did not breed in one of our nest
209 boxes. After implantation, two out of seven C females and four out of seven T females moved to a
210 different nest box.

211 **2.3 Incubation measurements**

212 As soon as a female was observed incubating, the temperature of her nest was measured, from which
213 incubation temperature and incubation behaviour was calculated. We used a data logger (HOBO logger,
214 Mulder-Hardenberg BV., The Netherlands) that registered the temperature inside the nest box via a
215 sensor that was positioned in the middle of the nest on the first day of incubation. To place the sensor,
216 the eggs were temporarily removed and a small hole was drilled in the bottom of the nest box. Through
217 this hole a sensor was mounted in the middle of the nest cup. The logger was stored in a small green
218 plastic box, which was taped to the lid of the nest box on the outside (Figure 1A). After mounting the
219 sensors, the eggs were placed back into the nest box, around the sensor (Figure 1B). The sensor did not
220 extend above the eggs. Drilling and placing the equipment did not take more than 10 min. The
221 temperature was measured every 15 s for an average period of 9967 ± 688 min from the onset of
222 incubation until eggs had hatched, and the measurement time did not significantly differ between
223 treatments (independent t-test $t_{11} = -0.04$, $P = 0.96$). To validate whether temperature fluctuation
224 recorded by the data logger coincided with presence or absence of the incubating female, a video
225 camera was placed approximately 5 m away from five nest boxes to record when a female entered or
226 left the nest box and when a male entered the nest box to feed the female. Before the start of each
227 video recording, the nest box was checked to see if the female was on the nest. A total of 264 min of
228 video observations were made with an average of 53 ± 4.5 min per nest, and presence and absences
229 recorded on video were afterwards visually compared to the temperature data plotted against time per
230 female. Gaps in incubation due to female absences (recorded via video) corresponded to a sharp decline
231 in temperature, and for the presence of females on eggs showed the opposite pattern. Once the
232 temperature data were validated, we developed threshold temperature values to identify presence and
233 absence of females. A sharp decline in temperature of more than 1.3 °C/min for at least 4 min was
234 considered as a gap in incubation. An increase in temperature with an initial slope of at least 0.2 °C/min
235 and a maximum slope of at least 1.0 °C/min was considered the start of a bout of incubation. These
236 criteria were used to automatically identify incubation bouts and gaps in the program Rhythm 1.0
237 (Cooper and Mills, 2005). Subsequently, we visually inspected these intervals with the program Raven
238 Pro (1.4) and manually corrected obvious errors (see also Cooper and Mills 2005, for the selection
239 procedure using Rhythm and Raven). We found in particular that the start of the recesses needed to be
240 corrected manually (most of the time by a few minutes) because the drop in temperature was relatively
241 slow in the beginning and only gradually became steep, perhaps because in a nest box species
242 temperature changes are relatively slow compared to non-hole breeding species. Therefore Rhythm did
243 not always select the complete recess period. From the identified time intervals of incubation bouts and
244 gaps, we calculated the following parameters; (i) duration of every incubation bout (min); (ii) duration of
245 every incubation gap (min); (iii) minimum nest temperature during a gap (°C); (iv) mean nest
246 temperature during the day (combining incubation bouts and gaps) (°C); and (v) mean nest temperature
247 during night (°C). The start of the night was defined from when the females started incubating in the
248 evening for a period longer than 2 hours until there was a sharp decline in temperature, when the
249 female exited her nest box in the morning. For the analyses of the incubation data we used data of 13
250 females (C=6; T=7). In the analyses we included the average time (min) a female spent off and on her
251 nest during the day, the number of gaps and the average minimum temperature during incubation gaps,
252 the overall mean incubation temperature during the day, the mean variation in temperature during the
253 day, and mean night temperature inside the nest.

254 **2.4 Natural hormone concentrations**

255 To examine the natural profile of circulating T levels, female blood was collected just prior to
256 implantation (n = 17) and from five C females that were recaptured during the nestling period (between
257 the 2nd and 15th of May 2009). None of the recaptured females lost their implant. During these
258 captures, 50 – 150 µl blood was taken within 30 min after capture by puncturing the brachial vein with a
259 sterile needle (Terumo, 27 g × 3/4; 0.4 × 20 mm) and transferred into an Eppendorf tube using
260 heparinized microhematocrit capillaries. The blood was stored on ice and centrifuged for 10 min at 7000
261 rpm within six hours after sampling. The plasma fraction was removed and stored at –20°C until analysis.
262 Testosterone was quantified in plasma extracts by radioimmunoassay (RIA) using a commercial double
263 antibody system purchased from MP Biomedicals (Solon, Ohio). For extraction, 500 µl of a 50/50 mixture
264 of cyclohexane/ethylacetate was added to 50 µl plasma and the tubes were incubated for 10 min with
265 continuous shaking. After centrifugation, the tubes were placed in a mixture of dry ice and ethanol for
266 snap freezing, followed by transfer of the organic phase to a new tube. After thawing, samples were re-
267 extracted following the same method. The combined supernatants were dried by vacuum centrifugation
268 and stored at –20°C until further analysis. For testosterone measurements, the dried samples were
269 dissolved in 25 µl steroid diluent buffer and further treated following the protocol of the RIA kit. The
270 primary antibody used in this assay does not significantly cross-react with other androgens beside T (5α-
271 dihydrotestosterone: 3.4%; 5α-androstane-3β, 17β-diol: 2.2%; 11-oxo-testosterone: 2%; all other
272 steroids: <1%). Testosterone standards ranged from 0.10 ng/ml to 11.75 ng/ml, but the effective
273 detection limit could be extended to 0.05 ng/ml owing to the concentration effect of the extraction
274 procedure. All samples were measured in a single assay and the intra-assay coefficient of variation was
275 4.6 for medium/low and 9.1% for high concentrations.

276 **2.5 Test of hormone implants**

277 Because we did not recapture enough T females (see table 1) to examine the effects of the implants on T
278 plasma concentrations, an additional laboratory experiment was conducted. In this experiment eight
279 female great tits were implanted with T on the 10th of December 2012. The females were implanted in
280 December, because during this non-breeding period female birds generally have very low T levels
281 (reviewed by Ketterson et al., 2005). Thus, an increase in T levels would most likely be caused by the
282 implants. The great tits were wild-caught and hand-reared and all the same age (2 years). Before and
283 during the first 14 days of the experiment these females were housed in single-sex groups of eight
284 individuals in free-flight, half-open aviaries (2.0 × 4.0 × 2.5 meters). After 14 days, four of the eight
285 females were housed together with a male in separate aviaries. Birds were provided with *ad libitum* food
286 and fresh water at all times. To measure baseline T plasma concentrations, a blood sample was taken
287 prior to implantation within 10 min after capture. Next the females were implanted with silastic tubes
288 filled with T. The implantation procedure and the implants used were equal to the field experiment (see
289 for size above; average T weight 0.6 ± 0.01 mg). The birds returned to their aviary within 90 min after
290 capture. Seven and 28 days after implantation another blood sample was taken to measure the effects of
291 the implants on T plasma concentrations. All the blood samples were taken between 12:00 –14:00
292 GMT+1. Immediately after sampling the blood was centrifuged for 10 min at 6000 rpm, the plasma was
293 removed, and stored at –20°C. After the last blood sample was taken the implants were removed under

294 local anaesthesia (Xylocaine, 10% spray) by making a small incision below the implant, and the incision
295 was sutured with tissue glue (1 × 0.5 ml Histoacryl, Braun, Germany). During each of the captures the
296 health of the females was checked by measuring their weight to the nearest 0.1 g. There was no
297 significant weight change over the course of the experiment (One-way repeated measures ANOVA: $F_{1,14} =$
298 2.26, $P = 0.15$).

299 The plasma samples were analysed in one assay using a commercial kit (Orion Diagnostica, Spectria
300 Testosterone RIA kit, Espoo, Finland) with a sensitivity of 0.04 ng/ml testosterone and cross-reactivities
301 of 4.5 % with DHT and 0.01 % with A4 as described in de Jong et al. (2013). In brief; plasma samples were
302 defrosted, their volume was measured and 50 µl radio-actively labelled testosterone (Perkin Elmer Life
303 and Analytical Science BV) was added to all samples to measure the accuracy of the extraction process
304 (recovery). After an incubation time of 1 hour, 2.5 ml diethyl ether/petroleum benzene (70:30) was
305 added and samples were vortexed and centrifuged. Samples were snap frozen by a mixture of ethanol
306 and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was
307 again dissolved in 1 ml 70% methanol and samples were stored over night at -20°C . The following day,
308 samples were centrifuged, the methanol phase was decanted and the samples dried again under
309 streaming nitrogen. The pellet was re-suspended in 200 µl PBS buffer. 30 µl of this mixture was used for
310 measuring recoveries (average recovery rate for testosterone: $92.96 \pm 0.89\%$). Hormone concentrations
311 were measured using radio immuno assays (RIAs). Based on the standard curve values below the
312 detection limit was calculated as being 0.10 ng/ml. The dilution curve ran parallel to the standard curve.
313 The intra assay variation was 6.9 %.

314 **2.6 Statistical analyses**

315 All the data were checked for normality. Data that were not normally distributed (number of days spent
316 incubating, incubation bouts and gaps, and fledging date) were transformed to approximate normality
317 when possible (see below) or tested using non-parametric tests. Independent t-tests were used to
318 analyse the effect of treatment on female characteristics (body mass (g), tarsus (mm) and wing length
319 (mm), the onset of egg laying and incubation date, clutch size, and the number of hatchlings and
320 fledglings. The effect of treatment on hatching date was analysed with a general linear model (GLM).
321 Treatment was included as a fixed factor and the onset of egg laying was included as a covariate.

322 To test whether T implants had an effect on T plasma levels of captive females 7 and 28 days after
323 implantation, a linear mixed model (MIXED) was used. The model included T plasma levels as a
324 dependent variable, sample period (where sample period two = 7 days after implantation; sample period
325 three = 28 days after implantation) as a fixed factor and female ID as a random factor. To correct for
326 possible disturbance effects from capturing on T levels, time between entering the aviary and blood
327 sampling was included as covariate. For the four birds that were housed separately during the last blood
328 sampling, the time between capture and blood sampling was taken. The housing condition of the
329 females when the third sample was taken had no effect on the hormone levels (independent t-test $t_5 =$
330 0.85, $P = 0.43$) and was therefore not included in the analyses.

331 The number of incubation gaps, the mean duration of incubation bouts and gaps, mean minimum
332 temperature, mean day temperature and mean variance in day temperature, and mean night

333 temperature were analysed with a GLM. Treatment was included as a fixed factor and clutch size was
334 included as a covariate in all models, as females with larger clutches spent more time inside their nest
335 boxes ($F_{1,12} = 8.37$, $P = 0.02$). The mean incubation gap duration and time spent incubating were not
336 normally distributed and were transformed with a \log_{10} transformation. Linear regression models were
337 used to quantify the relationship between the average incubation temperatures during the day or during
338 the night and the proportion of hatching success (the number of hatchlings divided by clutch size) per
339 nest, for each treatment separately (variance in hatching success was not equally distributed between
340 treatments).

341 To test the effect of treatment on hatching and fledging success, and nestling survival until day 6, a
342 generalized linear mixed model (GLIMMIX) was used, with a binary distribution and a logit function. An
343 egg was scored as 1 when it hatched and as 0 when it did not hatch. A nestling was scored as 1 when it
344 had survived until day 6 or fledged and 0 when it had not survived until day 6 or fledged. In the models
345 treatment was included as a fixed factor and female ID as a random factor. Clutch size was included as a
346 covariate to correct for initial differences in number of eggs. To test if the treatment of the mother had
347 an effect on nestling body mass (g) at day 10 and day 15, growth rate (measured as the change in body
348 mass between day 10 and 15 in g) and tarsus length (mm) at day 15, a linear mixed model was used,
349 including treatment as fixed factor, nest box as a random factor (to correct for non-independence of
350 nestlings of the same mother), date of measurement (for body mass), or hatching date (for hatchling
351 growth) and brood size as co-variables. Tests were two tailed and differences were considered to be
352 significant with a P-value <0.05 . SAS (SAS® 9.2) was used to analyse the nestling characteristics and
353 incubation data. All the other data were analysed using STATISTICA 7.0 (StatSoft, Inc.). Unless stated
354 otherwise, average values are presented \pm SEM while median values are presented \pm range.

355

356 **3. RESULTS**

357 **3.1 Hormone concentrations**

358 The mean natural T plasma concentration of female great tits during the nest building phase was $1.02 \pm$
359 0.30 ng/ml with a range of 0.10 – 2.67 ng/ml. During the nestling phase, the mean natural T concentration
360 of C females was 0.54 ± 0.07 ng/ml with a range of 0.31 – 0.67 ng/ml (see Fig. 2A). The average baseline T
361 level of the captive female great tits was 1.92 ± 0.49 ng/ml (see Fig. 2B). T levels differed significantly
362 between the three sampling periods (MIXED; $F_{2,11} = 9.42$, $P = 0.0041$, correcting for time: $F_{1,11} = 9.96$, $P =$
363 0.009). T levels during the first sampling period were significantly lower than during the second and third
364 sampling period (1 vs 2: Tukey test; $P = 0.004$; 1 vs 3: Tukey test; $P = 0.012$), while the latter two periods
365 did not differ significantly from each other (Tukey test; $P = 0.99$). The mean plasma T value after 7 and 28
366 days were 3.29 ± 0.39 ng/ml and 2.81 ± 0.22 ng/ml, respectively. As the T-implant induced values were
367 around the same values as some of the baseline values before implantation, the implant did not induce
368 supra-physiological values. Moreover, T concentrations during implantation did not return to the
369 average baseline level, indicating that the implants worked over a long period. The higher levels in winter
370 compared the average natural T levels during the nest building phase may be due to the fact that in this
371 species females compete during winter and form dominance hierarchies in winter flocks (Gosler, 1993).

372 This was clearly expressed in our aviaries where there was obvious competition (personal observation
373 KvO).

374 **3.2 Breeding parameters**

375 There was no effect of the T treatment on time it took females to complete the nest compared to those
376 from the control group (Table 2). T females laid their eggs on average four days later than C females;
377 however this difference was not significant. Clutch sizes of T and C females did not differ (Table 2).

378 **3.3 Incubation behaviour**

379 Treatment had a significant effect on incubation temperature; both the mean minimum temperature
380 inside the nest box during a gap in incubation (Fig. 3A) and the mean day and night temperatures (Fig.
381 3B, C) were significantly lower in the T than in the control group (Table 3). Mean variance in day
382 temperature was not affected by treatment, but clutch size did show a significant negative relationship
383 with mean variance in day temperature. Females with large clutches showed less variation in day
384 temperature, perhaps because eggs buffer each other against temperature changes. Mean day and night
385 temperatures were positively correlated with hatching success in T females whereas mean day
386 temperature was negatively correlated with hatching success in C females (Table 4). All other parameters
387 measured were not significantly different between the treatments (Table 3).

388 **3.4 Reproductive success**

389 The incubation time in days (C = 12; 12-13; T = 13; 13-14) was not significantly different between the two
390 treatments (Mann-Whitney U-test (1) = 7, Z = -1.71, P = 0.9). The average hatching date of nestlings ($66 \pm$
391 1.34) from T females was five days later than that of offspring produced by control females (61 ± 1.41),
392 but this effect was driven by a delay in egg laying as hatching date was not significantly different when
393 correcting for the onset of egg laying (treatment: $F_{1,9} = 0.66$, $P = 0.45$; onset of egg laying: $F_{1,9} = 12.45$, $P =$
394 0.006). Hatching success was significantly lower in the T treated group (GLIMMIX; hatching success: $F_{1,109}$
395 $= 4.01$, $P = 0.048$; clutch size; $F_{1,109} = 1.91$, $P = 0.17$). A total of 35 out of 52 (64%) eggs from T females
396 hatched compared to 68 out of 70 (97%) eggs from C females. After hatching, a higher proportion of
397 nestlings of T females did not survive until day 6 (10 out of 35) compared to those of C mothers (1 out of
398 68; GLIMMIX; nestling survival until day 6: $F_{1,109} = 8.14$, 0.0005 ; clutch size: $F_{1,109} = 0.08$, $P = 0.78$). Brood
399 size at day 6 after hatching was also significantly smaller in the treatment group compared to the control
400 group (Table 5.1). After day 6 nestling survival was almost equal in both groups, only one T nestling died
401 after day 15. But because the number of hatchlings and the number of nestlings surviving to day 6 were
402 lower for T females, the overall fledging success was significantly lower in the T treatment group
403 (GLIMMIX; fledging success: $F_{1,109} = 9.08$, $P = 0.003$; clutch size: $F_{1,109} = 0.08$, $P = 0.78$). A total of 24
404 fledglings from six T nests fledged, compared to 67 fledglings from seven control nests, which was
405 statistically significant (Table 2). The average fledging date of the nestlings did not differ between
406 treatments. In summary, prolonged increased levels of testosterone significantly reduced reproductive
407 success.

408 **3.4 Nestling characteristics**

409 On day 10, the average body mass of nestlings produced by T females (12.76 ± 0.45 g) was significantly
410 lower than that of nestlings produced by C females (13.39 ± 0.22 g) correcting for brood size (MIXED;
411 treatment: $F_{1,89} = 9.54$, $P = 0.003$; brood size: $F_{1,89} = 9.62$, $P = 0.003$). Nestlings of larger broods were on
412 average lighter. The date at which 10 day old nestlings were measured did not have a significant effect
413 on their weight (MIXED; date: $F_{1,88} = 2.57$, $P = 0.11$). There was no difference in weight between 15 day
414 old nestlings of T females (16.62 ± 0.27 g) and nestlings of C females (16.59 ± 0.18 g, MIXED; treatment:
415 $F_{1,88} = 0.45$, $P = 0.51$; date: $F_{1,88} = 3.43$, $P = 0.07$). The covariate brood size was negatively associated
416 with the weight of 15 day old nestlings (MIXED; brood size: $F_{1,88} = 5.57$, $P = 0.02$). Between day 10 and day
417 15, T nestlings (3.85 ± 0.28 g) grew significantly faster than C nestlings (3.20 ± 0.16 g, MIXED; treatment:
418 $F_{1,88} = 25.11$, $P < 0.0001$; hatching date: $F_{1,88} = 26.44$, $P < 0.0001$; brood size: $F_{1,88} = 7.82$, $P = 0.006$). Nestlings
419 that hatched later in the breeding season and/or grew up in larger broods showed a reduced growth.
420 Average nestling tarsus length did not differ between treatments (T nestlings: 19.61 ± 0.17 mm; C
421 nestlings: 19.48 ± 0.11 mm, MIXED; treatment: $F_{1,88} = 0.14$, $P = 0.71$) and was not affected by brood size
422 or date of measurement (MIXED; brood size: $F_{1,88} = 1.21$, $P = 0.279$; date: $F_{1,88} = 2.89$, $P = 0.09$). In
423 summary, females treated with prolonged elevated T levels had lighter chicks in the beginning of the
424 rearing period, but these chicks caught up in body mass thereafter.

425

426 4. DISCUSSION

427 In some avian species, elevation of male T after the formation of a pair and a territory can still increase
428 male reproductive success by mate guarding, territorial defence and obtaining extra pair offspring.
429 However, the hormone can also suppress parental care. To circumvent the detrimental effects of T on
430 male parental behaviour, certain species appear to have evolved insensitivity to T during the period of
431 intensive parental care (the essential paternal care hypothesis: Lynn et al 2002, Lynn 2008). These
432 species are generally species where male care is essential for offspring survival (for example the great tit,
433 Bjorklund and Westland, 1986a). Alternatively, behaviour may remain sensitive to elevated T levels,
434 when T-mediated behaviour is important for reproductive success even after egg laying, although this
435 might be detrimental for other reproductive behaviours (the essential mating effort hypothesis, Rosvall
436 2013a,b). Although behavioural insensitivity to T after egg production has been mainly tested in males, it
437 may be even more important for females that provide often most of parental care and suppressive
438 effects of T on female care is well documented (see introduction). We studied this in female great tits,
439 finding that, despite limited sample size and some multiple testing, several aspects of female parental
440 care are still sensitive to T, resulting in poorer care with negative effects on fitness.

441 Our findings correspond with those of a recent study on female tree swallows (Rosvall 2013a), T
442 treatment in these birds resulted in more aggression, poorer incubation and lower hatching success of
443 the chicks. This was explained by the fact that female tree swallows need T to defend their nest after egg
444 production and during chick rearing, even though this decreases offspring production. . However, in
445 great tits, females do not need to defend territories after the start of incubation. Therefore there would
446 be no need for T elevation as this would have detrimental effects on parental care and no clear benefits.
447 Indeed, endogenous T levels are low after the start of egg laying in this species (Rost, 1990). Therefore,
448 the fact that females remain sensitive to elevated T levels later in the breeding season, are in line with
449 our hypothesis that there has been no need for strong selection in the past in favour of becoming
450 insensitive to T in great tits.

451 4.1. Nest building behaviour

452 Many female bird species show a peak in T levels at the beginning of their breeding season, when
453 females are building their nests. Therefore we were surprised to find that elevated T did not affect nest
454 building behaviour. Only a few other studies have investigated the role of T in nest building behaviour
455 and the results are inconsistent. In the European starling, T did not affect nest building (De Ridder et al.,
456 2002), whereas in the blue tit, T females accelerated nest building (de Jong, 2013). The absence of a
457 treatment effect in our study might be due to the fact that we only had a somewhat rough classification
458 of nest completeness, and that we did not observe actual nest building behaviour.

459 4.2. Incubation behaviour

460 We found particularly strong negative effects of elevated T late in the breeding cycle, with T females
461 producing significantly lower incubation temperatures than C females. So far, two other studies have
462 investigated the effect of elevated T on incubation behaviour in female birds. Experimental elevation of T
463 levels did not affect the total time females spent incubating in dark-eyed juncos (Clotfelter et al., 2004),

464 but did decrease incubation temperature in the tree swallow (Rosvall, 2013a). A likely explanation for
465 the reduction in incubation temperature may be that T females had a less developed brood patch
466 (Clotfelter et al., 2004), reducing the maximum temperature a female can reach during incubation
467 (yellow-eyed penguin (*Megadyptes antipodes*) Massaro et al., 2006). Further research is required to
468 confirm this, as this effect of T on the brood patch could be one of the functional explanations for why T
469 levels dropped quickly when females start incubating.

470 **4.2.1 Incubation behaviour and hatching success**

471 The lower incubation temperature in the nest of T females was inversely correlated with the hatching
472 probability of their eggs. Egg temperature during incubation is important for the development of the
473 embryo (Webb, 1987) and low incubation temperature can cause mortality of the embryos before
474 hatching (Deeming and Ferguson, 1991), decrease hatching success (blue tit, Nord and Nilsson, 2011),
475 increase nestling mortality (domestic white leghorn chicken (*Gallus gallus*), Evans, 1990), or decrease
476 nestling weight (domestic white leghorn chicken, Suarez et al., 1996; tree swallow, Ardia et al., 2010).
477 Therefore the lower hatching success of eggs of T females compared to C females might be causally
478 explained by the low incubation temperature in the former. As a consequence, females treated with T
479 produced fewer hatchlings and fledglings and therefore had a lower reproductive success. A reduction in
480 hatchling and/or fledgling number due to elevated T in their mothers has been shown in other bird
481 species too (O'Neal et al., 2008; Lopez-Rull and Gil, 2009; Veiga and Polo, 2008). Surprisingly, C females
482 that showed higher mean incubation temperatures during the day, also showed lower hatching success.
483 In fowl (*Gallus gallus domesticus*), very low and very high incubation temperature have both been shown
484 to negatively affect embryonic development, indicating that there is an optimum incubation
485 temperature for embryos to develop normally (Romanoff et al., 1938). Since in our study the nests with
486 the lowest and the highest incubation temperature had lower hatching success, this might have been
487 caused by a deviation from such an optimum incubation temperature in these nests. Alternatively, T
488 implantation resulted not only in higher circulating concentration of the hormone in the female but also
489 in her eggs, with the latter negatively affecting hatching success. Although there is evidence that female
490 birds can independently regulate T concentrations in their circulation from those in her eggs (Japanese
491 quail (*Coturnix japonica*), Okuliarova et al 2011), T implantations can indeed lead to higher T
492 concentrations in the egg (Groothuis and Schwabl 2008). However, from the many *in ovo* T injection
493 studies there is no evidence at all that elevated yolk T negatively affects hatching success (Von
494 Engelhardt and Groothuis 2011).

495

496 **4.3. Nestling phase**

497 At day 10, T nestlings had lower body masses compared to C nestlings. There are three possible
498 (mutually not exclusive) explanations why nestling weight was affected by the T treatment. First, eggs
499 laid by T females may have been lighter and therefore produced lighter nestlings. Great tit nestlings that
500 hatch from lighter eggs grow more slowly during the early period of the hatching phase, but do catch up
501 before fledging (Schifferli, 1973). However, effects of T treatment of females on egg mass are
502 ambiguous; studies have either found no effect of T on egg mass (Clotfelter et al., 2004; Lopez-Rull and

503 Gil, 2009), or an increase in egg mass (Rutkowska et al., 2005). A pilot study on the effects of T on egg
504 mass in great tits, however, did show a decrease in egg mass (R. Pinxten, unpubl. data).

505 Second, a lower brooding temperature of the mother might have affected the weight of the nestlings. A
506 previous study in dark-eyed juncos showed that females treated with T indeed showed reduced brooding
507 of young (O'Neal et al., 2008). Brooding behaviour of the mother is very important for nestlings because
508 thermoregulation of altricial young is not yet fully developed in the early nestling stage (Dunn, 1975).
509 Thus in our study female brooding may have resulted in chicks having to spent more energy on
510 thermoregulation and less on energy for growth and development, resulting in lower weight. This is
511 consistent with the fact that chick survival was lower of T nestlings during the first 6 days compared to
512 the period of the nestling phase after day 6. After 6 days, nestlings can regulate their own temperature
513 and therefore are less dependent on the brooding of the mother.

514 Third, nestling body mass may have been affected by differential food provision rates of T mothers. In
515 the spotless starling, T treatment of females reduced female feeding rate (Veiga and Polo, 2008),
516 whereas in dark-eyed juncos there was no difference in feeding rate between T and C females (O'Neal et
517 al., 2008). Feeding rates were not quantified in this study, but if feeding behaviour of T females was
518 affected negatively, then this most likely only occurred during the early nestling period since nestling
519 weight did not differ anymore on day 15. The fact that the chicks from T treated mothers caught up in
520 weight after day 10 may be caused by increased maternal provision. This has been found in female dark-
521 eyed junco's in which those with natural high T levels brooded less but provisioned nestling more
522 frequently (Cain and Ketterson, 2013). Alternatively, elevated T concentrations in the egg might have
523 induced higher growth rates, as found in several avian studies (reviewed by Von Engelhardt and
524 Groothuis 2011).

525 **5. Conclusions**

526 Overall, our results indicate that great tit females are behaviourally sensitive to elevated T levels during
527 the period of maternal care, at the cost of incubation behaviour and hatching success of their offspring.
528 Since in this period natural T levels are normally low we suggest that Darwinian selection for T
529 insensitivity in great tits has not been strong because it was never needed. One potential restriction in
530 our study was that we only measured parental care and not aggressive behaviour, in contrast to an
531 earlier study on female tree swallows (Rosvall 2013a), which also found behavioural sensitivity to T after
532 egg laying. However, females of our study species have less need to show aggression after egg laying. We
533 hypothesise that in species in which females do need T induced aggressive behaviour during the phase of
534 maternal care, the suppressive cost of T on this care are avoided either by much more moderate levels of
535 T than induced by implantation studies, or by becoming T insensitive in maternal care but not in
536 aggressive behaviour. That such behaviour-specific regulation can occur has been demonstrated in males
537 in several species. This includes males of our study species, in which T implantations induce higher song
538 rates but not at the cost of reduced parental care (Van Duyse et al. 2002).

539 **Funding**

540 BDJ was supported by a doctoral grant from the Flemish Agency for Innovation by Science and
541 Technology.

542 **Acknowledgments**

543 We thank Peter Scheys and Ann Geens for field assistance, and Bonnie de Vries and Lut Noterdaeme for
544 lab assistance. We thank Lewis Spuring for comments on the manuscript. The study was conducted in
545 full compliance with Belgian and Dutch laws and regulations.

546

547 **REFERENCES**

- 548 Adkins-Regan, E., 2005. Hormones and animal social behavior. Princeton University Press, Princeton
- 549 Alonso-Alvarez, C., 2001. Effects of testosterone implants on pair behaviour during incubation in the
550 Yellow-legged Gull *Larus cachinnans*. *Journal of Avian Biology* 32, 326–332.
- 551 Ardia, D.R., Perez, J.H., Clotfelter, E.D., 2010. Experimental cooling during incubation leads to reduced
552 innate immunity and body condition in nestling tree swallows. *Philosophical Transactions of the Royal
553 Society B-Biological Sciences* 277, 1881–1888.
- 554 Bjorklund, M., Westman, B., 1986a. Mate-guarding in the great tit - tactics of a territorial forest-living
555 species. *Ornis Scandinavica* 17, 99–105.
- 556 Björklund, M., Westman, B., 1986b. Adaptive advances of monogamy in the great tit (*Parus major*): an
557 experimental test of the polygyny threshold model. *Animal Behaviour* 34, 1436-1440.
- 558 Cain, K.E., Ketterson, E.D., 2012. Competitive females are successful females; phenotype, mechanism,
559 and selection in a common songbird. *Behavioral Ecology and Sociobiology* 66, 241–252.
- 560 Cain, K. E., Ketterson, E.D., 2013. Individual variation in testosterone and parental care in a female
561 songbird; the dark-eyed junco (*Junco Hyemalis*).” *Hormones and Behavior* 64, 685–92.
- 562 Clotfelter, E.D., O’Neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L.,
563 Nolan, V., Ketterson, E.D., 2004. Consequences of elevating plasma testosterone in females of a socially
564 monogamous songbird: evidence of constraints on male evolution? *Hormones and Behavior* 46, 171–
565 178.
- 566 Cramp, S., Perrins, C.M., 1993. *Parus major* Great tit, in: *Handbook of the birds of Europe, the Middle
567 East and North Africa. The birds of the western palearctic*. Oxford University Press, Oxford, pp. 255–281.
- 568 De Jong, B., 2013, Testosterone a female hormone – Testing the function and evolution of testosterone
569 in female birds. University of Groningen.
- 570 De Ridder, E., Pinxten, R., Eens, M., 2000. Experimental evidence of a testosterone-induced shift from
571 paternal to mating behaviour in a facultatively polygynous songbird. *Behavioral Ecology and Sociobiology*
572 49, 24–30.
- 573 De Ridder, E., Pinxten, R., Mees, V., Eens, M., 2002. Short- and long-term effects of male-like
574 concentrations of testosterone on female European starlings (*Sturnus vulgaris*). *Auk* 119, 487–497.
- 575 Deeming, D.C., Ferguson, M.W.J., 1991. *Egg Incubation: Its effects on embryonic development in birds
576 and reptiles*. Cambridge University Press, Cambridge.
- 577 Dunn, E., 1975. Timing of endothermy in development of altricial birds. *Condor* 77, 288–293.

578 Edler, R., Goymann, W., Schwabl, I., Friedl, T.W.P., 2011. Experimentally elevated testosterone levels
579 enhance courtship behaviour and territoriality but depress acquired immune response in Red Bishops
580 *Euplectes orix*. Ibis 153, 46–58.

581 Enstrom, D.A., Ketterson, E.D., Nolan, Jr, V., 1997. Testosterone and mate choice in the dark-eyed junco.
582 Animal Behaviour 54, 1135–1146.

583 Evans, R.M., 1990. Effects of low incubation temperatures during the pipped egg stage on hatchability
584 and hatching times in domestic chickens and ring-billed gulls. Canadian Journal of Zoology 68, 836–840.

585 Gerlach, N.M., Ketterson, E.D., 2013. Experimental elevation of testosterone lowers fitness in female
586 dark-eyed juncos. Hormones and Behavior 63, 782–90.

587 Groothuis, T.G.G., Schwabl, H., 2008. Hormone-mediated maternal effects in birds: Mechanisms matter
588 but what do we know of them? Philosophical Transactions of the Royal Society B-Biological Sciences 363,
589 1647–61.

590 Gosler, A., 1993. The great tit. Hamlyn, London.

591 Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on
592 parental investment and breeding success in male house sparrows. Auk 104, 462–469.

593 Hunt, K.E., Hahn, T.P., Wingfield, J.C., 1997. Testosterone implants increase song but not aggression in
594 male lapland longspurs. Animal Behaviour 54, 1177–92.

595 Kazama, K., Sakamoto, K.Q., Niizuma, Y., Watanuki, Y., 2011. Testosterone and breeding behavior in male
596 black-tailed gulls: An implant experiment. Ornithological Science 10, 13–19.

597 Ketterson, E.D., Nolan, V., Sandell, M., 2005. Testosterone in females: Mediator of adaptive traits,
598 constraint on sexual dimorphism, or both? The American Naturalist 166, S85–S98.

599 Langmore, N.E., Cockrem, J.F., Candy, E.J., 2002. Competition for male reproductive investment elevates
600 testosterone levels in female dunnocks, *Prunella modularis*. Proceedings of the Royal Society of London
601 Series B-Biological Sciences 269, 2473–2478.

602 Lopez-Rull, I., Gil, D., 2009. Elevated testosterone levels affect female breeding success and yolk
603 androgen deposition in a passerine bird. Behavioural Processes 82, 312–318.

604 Lynn, S.E., 2008. Behavioral insensitivity to testosterone: Why and how does testosterone alter paternal
605 and aggressive behavior in some avian species but not others? General and Comparative Endocrinology
606 157, 233–240.

607 Lynn, S.E., Hayward, L.S., Benowitz-Fredericks, Z.M., Wingfield, J.C., 2002. Behavioural insensitivity to
608 supplementary testosterone during the parental phase in the chestnut-collared long-spur, - *Calcarius*
609 *ornatus*. Animal Behaviour 63, 795–803.

610 Nord, A., Nilsson J., 2011. Incubation temperature affects growth and energy metabolism in blue tit
611 nestlings. *American Naturalist* 178, 639–51.

612 Massaro, M., Davis, L.S., Davidson, R.S., 2006. Plasticity of brood patch development and its influence on
613 incubation periods in the yellow-eyed penguin *Megadyptes antipodes*: an experimental approach.
614 *Journal of Avian Biology* 37, 497–506.

615 Okuliarova, M., Grootuis, T.G.G., Skrobánek, P., Zeman, M., 2011. Experimental evidence for genetic
616 heritability of maternal hormone transfer to offspring. *The American Naturalist* 177, 824–34.

617 O’Neal, D.M., Reichard, D.G., Pavilis, K., Ketterson, E.D., 2008. Experimentally-elevated testosterone,
618 dark-eyed Junco (*Junco hyemalis*). *Hormones and Behavior* 54, 571–578.

619 Oring, L.W., Fivizzani, A.J., Elhalawani, M.E., 1989. Testosterone-induced inhibition of incubation in the
620 spotted sandpiper (*Actitis-Mecularia*). *Hormones and Behavior* 23, 412–423.

621 Raouf, S.A., Parker, P.G., Ketterson, E.D., Nolan, V., Ziegenfus, C., 1997. Testosterone affects
622 reproductive success by influencing extra-pair fertilizations in male dark-eyed juncos (Aves: *Junco*
623 *hyemalis*). *Proceedings of the Royal Society of London Series B-Biological Sciences* 264, 1599–1603.

624 Romanoff, A.L., Smith, L.L., Sullivan, R.A., 1938. Biochemistry and biophysics of the developing hen’s egg.
625 III. Influence of temperature. *Cornell University Agricultural Experimental Station Memoranda* 216, 1–42.

626 Rost, R., 1990. Hormones and behavior - a Joint examination of studies on seasonal-variation in song
627 production and plasma-levels of testosterone in the great tit *Parus major*. *Journal Fur Ornithologie* 131,
628 403–411.

629 Rosvall, K.A., 2013a. Life history trade-offs and behavioral sensitivity to testosterone: An experimental
630 test when female aggression and maternal care co-occur. *PLoS ONE* 8, e54120.

631 Rosvall, K.A., 2013b. Proximate perspectives on the evolution of female aggression: good for the gander,
632 good for the goose? *Proc. Trans R. Soc B* 368: 20130083

633 Rutkowska, J., Cichon, M., Puerta, M., Gil, D., 2005. Negative effects of elevated testosterone on female
634 fecundity in zebra finches. *Hormones and Behavior* 47, 585–591.

635 Sandell, M.I., 2007. Exogenous testosterone increases female aggression in the European starling
636 (*Sturnus vulgaris*). *Behavioral Ecology and Sociobiology* 62, 255–262.

637 Schifferli, L., 1973. The effect of egg weight on the subsequent growth of nestling great tits *Parus major*.
638 *Ibis* 115, 549–558.

639 Searcy, W.A., 1988. Do female red-winged blackbirds limit their own breeding densities? *Ecology* 69, 85–
640 95.

641 Slagvold, T., 1993. Female-female aggression and monogamy in great tits *Parus Major*. *Ornis*
642 *Scandinavica* 24, 155-158.

- 643 Suarez, M.E., Wilson, H.R., McPherson, B.N., Mather, F.B., Wilcox, C.J., 1996. Low temperature effects on
644 embryonic development and hatch time. *Poultry Science* 75, 924–932.
- 645 Svensson, L., 1984. Identification Guide to European Passerines. Stockholm.
- 646 Van Duyse, E., Pinxten, R., Eens, M., 2000. Does testosterone affect the trade-off between investment in
647 sexual/territorial behaviour and parental care in male great tits? *Behaviour* 137, 1503–1515.
- 648 Van Duyse, E., Pinxten, R., Eens, M., 2002. Effects of testosterone on song, aggression, and nestling
649 feeding behavior in male great tits, *Parus major*. *Hormones and Behavior* 41, 178–186.
- 650 Veiga, J.P., Polo, V., 2008. Fitness consequences of increased testosterone levels in female spotless
651 starlings. *American Naturalist* 172, 42–53.
- 652 von Engelhardt, N., Groothuis, T.G.G., 2011. Maternal hormones in avian Eggs. In hormones and
653 reproduction of vertebrates. Vol. volume 1. Academic Press.
- 654 Webb, D.R., 1987. Thermal tolerance of avian embryos: A review. *The Condor* 89, 874.
- 655 Wingfield, J.C., 1990. Hormonal-control of territorial behavior in Birds - field and laboratory
656 investigations. *Progress in Comparative Endocrinology* 342, 697–703.

657 **FIGURE LEGENDS**

658 **Figure 1. A)** Nest box with box attached to it. Inside the box was the data logger that recorded incubation
659 temperature. **B)** Great tit clutch with a sensor placed in between the eggs (see arrow).

660 **Figure 2. A)** Natural testosterone levels during the breeding season in female great tits. Open circles left
661 of the dotted line are individual pre-breeding T levels. Open circles to the right of the dotted line are T
662 levels during the nestling phase. The square is the average T level pre-breeding and the closed circle is
663 the average T level during the nestling phase (mean \pm SEM). **B)** The effect of T implants on plasma
664 testosterone of captive female great tits. Blood samples were taken prior to implantation (baseline
665 sample) and seven and 28 days after implantation. Each symbol represents the T-levels of one individual
666 female.

667 **Figure 3. (A)** The mean minimum incubation temperature during the recess of incubation. (B) Mean
668 incubation temperature during the day (including progresses and recesses of incubation). (C) Mean
669 incubation temperature during the night. Light grey bars are the control females, dark grey bars are the
670 testosterone females. Letters indicate a significant difference with $P < 0.05$. See table 2 for specific P-
671 values. Means \pm SEM are presented.

672

673 **Table 1.** Sample sizes used for the statistical analyses.

	Testosterone	Control	Total
Females implanted	12	9	21
Breeding in the population after implantation	7	7	14
Baseline T levels in the field	7	3	10 ¹
T levels after implantation	4	5 ¹	9
All breeding parameters until hatching	7	7	14 ²
Incubation measurements	6	7	13 ³
All parameters after hatching	7	6	13 ³
Captive birds	Baseline	Day 7	Day 28
T levels ⁴	8	7	7

674 ¹ Of the total of 17 females of which blood was sampled prior to implantation to measured natural T plasma
 675 concentrations seven individuals from the early breeding period were excluded because an insufficient amount of
 676 blood was collected for the hormone analyses. Also, two of the seven control females from the nestling period
 677 were excluded for that reason.

678 ² For the analyses of the different breeding parameters seven C and seven T nests were included until the onset of
 679 egg laying.

680 ³ During the incubation phase, one nest box of a T female was lost due to vandalism. Therefore seven C and six T
 681 birds were included in the analyses of the nestling phase.

682 ⁴ One female had lost her implant after implantation, therefore only seven of the 8 females were included for the
 683 analyses of the second and third sample.

684

685 **Table 2.** Summary of the overall treatment effects on different breeding parameters, nestling
 686 characteristics and reproductive output of female great tits. Data are presented as mean \pm SEM or as
 687 median (quartile range).

	Testosterone		Control		Test	
	Mean/ Median	SEM/ Quartile range	Mean/ Median	SEM/ Quartile range	T/U	P
Nest building time (days)	9.83	2.24	13.86	2.31	1.24 ²	0.24
Onset of egg laying¹	43	1.20	39.14	1.61	1.92 ²	0.08
Clutch size	8.71	0.52	10.00	0.82	1.33 ²	0.21
Number of hatchlings	5.83	1.38	9.71	0.87	2.46²	0.03
Brood size at day 6	4.17	1.42	9.57	0.75	3.44²	0.005
Number of fledglings per nest	4.00	1.37	9.57	0.75	3.72²	0.003
Fledging date¹	84	82-87	83	74-84	-1.23 ³	0.22

688 ¹The onset of egg-laying, hatching date and fledging date were scored in March days, where 1st of March
 689 is 1.

690 ²Independent t-test.

691 ³Mann-Whitney U-test.

692

693

694 **Table 3.** Summary of the overall treatment effects on incubation behaviour of female great tits. Data are
 695 presented as mean \pm SEM or as median (quartile range).

	Testosterone		Control		Test
	Mean/ Median	SEM/ Quartile range	Mean/ Median	SEM/ Quartile range	GLM
On-bout time	20.00	18.31-26.45	21.13	20.62-23.43	Treatment: F1,12, = 0.16, P = 0.70; Clutch size: F1,12 = 3.92, P = 0.08
Recess time	6.44	5.23-10.30	6.39	4.43-19.17	Treatment: F1, 12, = 0.12, P = 0.74; Clutch size: P > 0.45
Nr of recesses	185.14	33.52	181.33	21.46	Treatment: F1, 12, = 0.93, P = 0.36; Clutch size: P > 0.80
Minimum temperature during a recess	23.37	0.69	25.88	0.33	Treatment.: F1, 12, = 9.67, P = 0.01; Clutch size: P > 0.20
Day time temperature	28.62	0.79	31.23	0.44	Treatment.: F1, 12, = 7.59, P = 0.02; Clutch size: P > 0.70
Night time temperature	31.13	0.94	33.92	0.58	Treatment.: F1, 12, = 5.93, P = 0.03; Clutch size: P > 0.90

696
 697
 698

699 **Table 4.** Summary of the regression analyses of the relationship per treatment between incubation
700 temperature during the day/ night and hatching success.

	Testosterone				Control			
	Intercept	Slope	R ²	<i>P</i>	Intercept	Slope	R ²	<i>P</i>
Day temperature	-6.36	0.25	0.66	0.048	5.90	-0.14	0.80	0.02
Night temperature	-6.34	0.23	0.67	0.046	4.59	-0.09	0.59	0.08

701

702

703

704

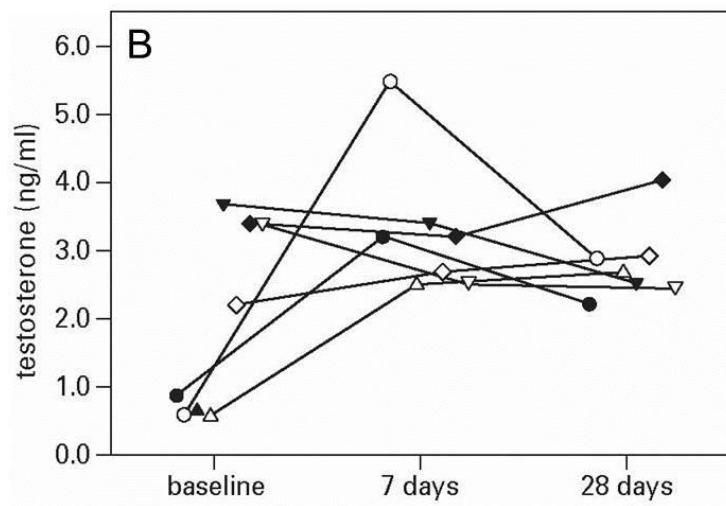
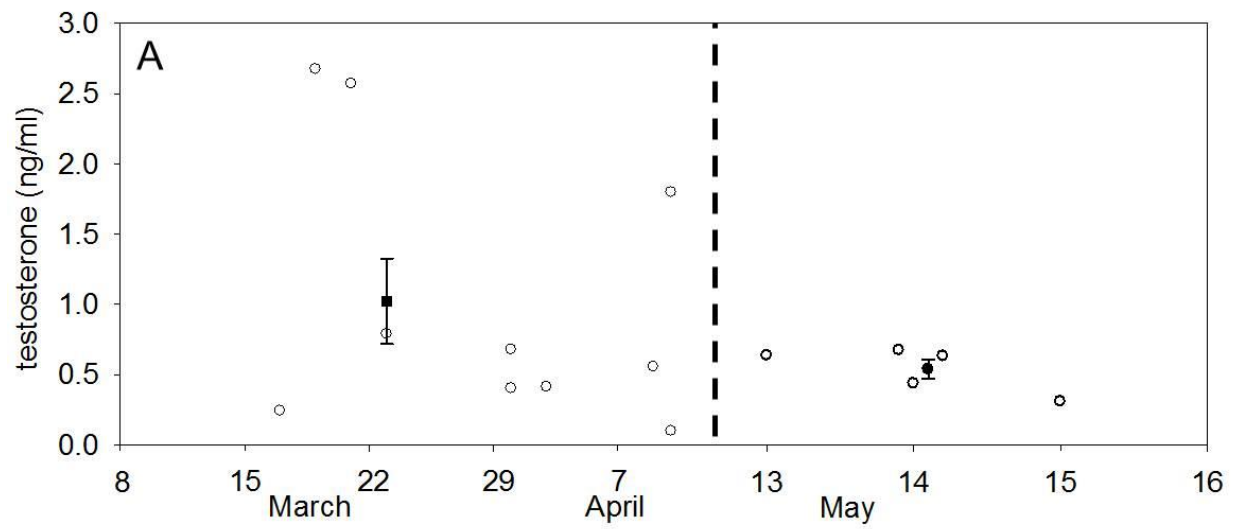
705



706

707 Figure 1.

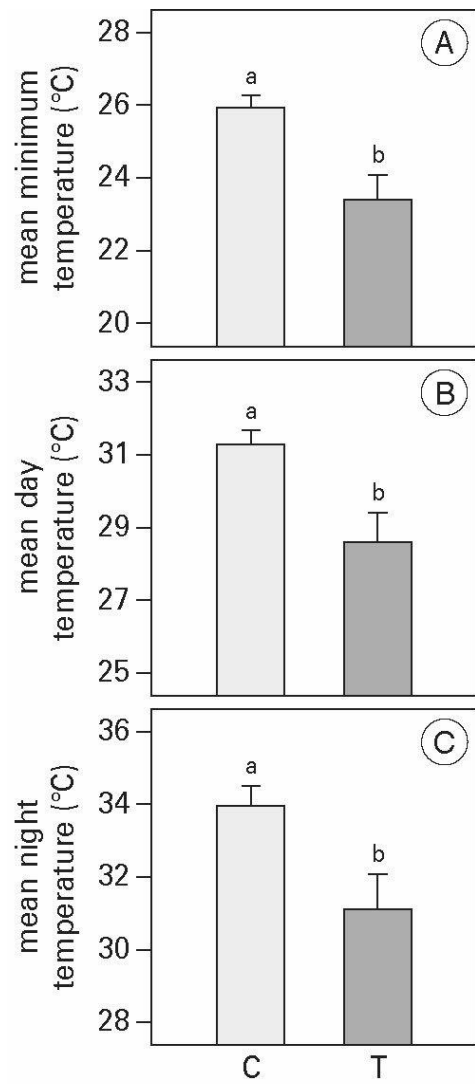
708



709

710 Figure 2A and 2B.

711



712

713 Figure 3.

714