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ORIGINAL ARTICLE

Life-long testosterone and antiandrogen treatments affect the survival and reproduction of captive male red-legged partridges (*Alectoris rufa*)

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Abstract

Sexual steroids can play an important role as life-history organizers. In males, high circulating testosterone levels induce physiological/behavioral costs and benefits, leading to trade-offs. However, studies simultaneously testing the impact of these levels in both fitness components (survival and fecundity) during lifetime are scarce and limited to wild birds. To determine the mortality causes or hormonal manipulation impacts on male fertility is, nonetheless, a difficult task in free-ranging animals that could be easier in captivity. We longitudinally monitored captive red-legged partridges (*Alectoris rufa*) and exposed males to high exogenous testosterone levels, anti-androgens, or a control treatment during each breeding period throughout their lives. Theory predicts that individuals maintaining high androgen levels should obtain higher fitness returns via reproduction, but suffer reduced longevity. Testosterone-treated male partridges, accordingly, lived shorter compared to controls, since they were more prone to die from a natural bacterial infection. However, the same birds seemed to have a lower capacity to fertilize eggs, probably due to endocrine feedback reducing testicular mass. These results show that exogenous testosterone can exert unpredicted effects on fitness parameters. Therefore, caution must be taken when drawing conclusions from non-fully controlled experiments in the wild. Males treated with the androgen-receptor blocker flutamide did not outlive controls as predicted by the life-history trade-off theory, but their mates laid eggs with higher hatching success. The latter could be due to mechanisms improving sperm quality/quantity or influencing maternal investment in egg quality. Testosterone receptor activity/amount could thus be as relevant to fitness as testosterone levels.

Significance statement

It has repeatedly been hypothesized that high testosterone levels induce a cost in terms of reduced lifetime reproductive success. This can be due to reduced fecundity or via shorter lifespan. This is, however, only supported by a handful of studies, mostly in wild birds. We tested this in captive male red-legged partridges, which allowed us to determine reproductive success and mortality causes. We increased testosterone levels or blocked its action with antiandrogens throughout life. High testosterone levels reduced the survival by making birds more prone to die by infection. The eggs produced by their mates also showed lower

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hatching success, a probable manipulation artifact that should be considered in avian studies in the wild. Interestingly, the androgen-receptor blocker flutamide increased lifetime hatching success compared to controls, suggesting that androgen receptor amounts/activity are even more relevant to fitness than testosterone levels.

Keywords Aging · Immunocompetence handicap · Oxidative cost of reproduction · Phenotypic engineering · Reproductive costs · Senescence

Introduction

The role of the circulating levels of androgens, particularly testosterone (T), on individual lifespan has been broadly studied in mammalian models. In humans, these hormone levels could, at least partially, explain the difference in longevity between sexes (reviewed in Brooks and Garratt 2017). Moreover, serum testosterone concentrations seem to show a quadratic inverse U-shaped relationship with human longevity (Yeap et al. 2014), and exogenous testosterone injections reduce lifespan in mice (Bronson and Matherne 1997), whereas castration extends life in rodents, primates, and humans (Asdell et al. 1967; Mills et al. 2009; Min et al. 2012; Zhang et al. 2015; Kessler et al. 2016). Therefore, testosterone is thought to exert a strong effect on male survival and, hence, lifespan.

Studies addressing the impact of testosterone levels in male fitness (i.e., not only considering survival but also fecundity) from an evolutionary point of view, however, are scarce and mostly restricted to avian species (but see Mills et al. 2009 for a study in wild rodents). In the case of the longevity component of fitness, the negative impact of sustaining high circulating testosterone levels on survival is well-supported by several studies in wild birds, where males subcutaneously implanted with exogenous testosterone (T-males hereafter) showed lower return rates (Dufty 1989; Nolan et al. 1992; Reed et al. 2006). Moreover, in red grouse *Lagopus scoticus*, similar experiments but involving radio-tracking monitoring (higher accuracy of survival estimation) detected a significant effect of testosterone implants on overwinter survival (Moss et al. 1994; Mougeot et al. 2005a). In the case of the fecundity component of fitness, also in red grouse, the testosterone treatment seems to indirectly increase reproductive success by allowing males to obtain better territories that favor overwinter survival (Moss et al. 1994). Some studies, however, suggest that testosterone could instead impair reproductive success. In both dark-eyed juncos (*Junco hyemalis*) and red grouse, authors suggested that an impairment of reproductive success was, at least partially, due to a potential suppressing effect of testosterone on parental care (Raouf et al. 1997; Reed et al. 2006; Martínez-Padilla et al. 2014a).

Most of the studies manipulating and testing the impact of high testosterone levels, however, involved only a short time period, since the implants usually run last only a few weeks or months. This procedure may contrast with scenarios where individuals experience high testosterone levels during repeated periods throughout a lifetime, probably as a consequence of high competitiveness in some populations (e.g., Mougeot et al. 2005a; Martínez-Padilla et al. 2014a). These scenarios could deeply affect the fitness outcomes of testosterone-mediated life-history trade-offs but have rarely been addressed in experimental setups.

To our knowledge, only the dark-eyed junco model has provided information in this regard. Among the many classical studies on this species, only Reed et al. (2006) describes an experiment where wild males were implanted with the same testosterone treatment during consecutive years, testing how the resulting artificial phenotype (see Ketterson et al. 1996 for the concept) behaved in terms of both reproductive output and survival. They showed that prolonged exposure to high testosterone levels reduced survival, but also obtained higher reproductive success thanks to extra-pair fecundity. The authors suggested that the net effect could, however, be negative as these males produced low quality (lower body mass gain) nestlings, probably due to reduced parental care. This conclusion was based on previous works where T-treated male juncos reduced feeding rates (Ketterson and Nolan 1992; Schoech et al. 1998). However, in other studies, T-treated male juncos showed a non-significant tendency to produce fewer nestlings than control males (Ketterson et al. 1996; Raouf et al. 1997). Such a reduction could be due to a lower capacity of T-treated males to fertilize eggs. This possibility was also argued by Foerster and Kempenaers (2004) and Martínez-Padilla et al. (2014a) when detecting reduced breeding success in T-treated male blue tits *Parus caeruleus* and red grouse, respectively (though they did not measure hatching success).

In the present study, to test the hypothesis that testosterone level/activity may determine main fitness outcomes, male red-legged partridges (*Alectoris rufa*) were manipulated each year during their successive breeding periods with the aim to produce different lifetime testosterone-mediated phenotypes. This experiment was performed in captivity, which assured to obtain accurate individual longevity and fecundity data. Extrapolating findings from experiments in captivity to scenarios under natural selection should be done carefully, as artificial environment conditions involve inherent limitations (see "Discussion"). However, considering these limitations, such experimental setups may ultimately clarify inconsistent findings described in wild bird studies (above). Thus, in this

study, birds were treated as controls (C-males) or with either testosterone (T-males), a blocker of androgen receptors (flutamide; "F"-males), or an inhibitor of testosterone conversion to estrogens (1,4,6-androstatriene-3,17-dione; ATD) plus flutamide (FA-males) during six consecutive breeding seasons. The FA-treatment aimed to block not only the direct receptor-mediated effects of T but those derived from the estrogen properties (e.g., Badeau et al. 2005), which may increase due to the aromatization of testosterone into estradiol when androgen receptors are blocked (Soma et al. 1999). These hormone treatments would maintain steady hormonal states during reproduction, which may contrast with short-term fluctuating situations in the wild (e.g., Wingfield et al. 1990; Apfelbeck et al. 2011). In terms of predictions, in the case of the longevity fitness component, we tentatively expected that T-males should live shorter than controls, but F- and FA-males would live longer. In terms of fecundity, we predicted that females of F- and FA-males should produce less offspring, whereas T-males should produce more.

Material and methods

Experimental protocol

The study was carried out at the Dehesa Galiana experimental facility (Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain). It was conducted on captive-born, 1-year-old (born in spring, 1 May 2005), red-legged partridges that had never bred before. Here we tested the same captive birds used in Alonso-Alvarez et al. (2008) and Cantarero et al. (2019) but analyzed the impact of testosterone treatments on longevity and breeding output. All individuals were given ad libitum access to commercial pelleted food and water. We used 117 randomly formed pairs that were placed in outdoor cages (1 × 0.5 × 0.4 m, length, width, height) at ambient temperature and natural photoperiod. Three males were excluded from the analyses (also excluded in the dataset) as they escaped during the first weeks of the experiment (N = 114). The partridges were acclimatized from outdoor aviaries to cages during 15 days, before starting the experiment (April). To minimize observer bias, blinded methods were used when all data were recorded and/or analyzed.

All males were blood sampled (see details in electronic supplementary material, ESM) and weighed on 10 April 2006 and subcutaneously implanted with hormonal treatments (see below) 10 days later. These dates coincide with the period of highest circulating luteinizing hormone (LH) levels in this species (Bottoni et al. 1993), a hormone that stimulates gonad testosterone secretion (e.g., Maung and Follett 1978). The same authors reported the highest testosterone concentrations in April (Bottoni et al. 1993). At the end of the egg-laying period (June 30), implants were removed, and 10 days later (July 10), all the birds were released in two large outdoor aviaries (46 × 8.5 × 3 m; length, width, height; 391 m^2 each one). These two outdoor aviaries were fully adjacent, only separated by a chain-link fence on the long side of the aviary (46 m). All treatments were represented and well-balanced in each aviary. Birds were recaptured from outdoor aviaries in mid-March of the following year. This chronogram was repeated yearly until 2011 (see also Cantarero et al. 2019). The same hormonal treatment (see below) was maintained for each bird during the lifetime. No bird was implanted in 2012 as no control bird was alive in that breeding period. Nonetheless, the survivorship of the remaining three birds (one FA- and two F-males) was recorded until January 2013 when the last (almost 8 years old) bird died. For breeding, males were paired with the same female partner in all breeding events unless the female died or was injured by male pecking and hence removed from the experiment. In these cases, the female was replaced by a yearling female from another non-manipulated captive pool. The occurrence of pecking throughout lifetime (in 20 males, i.e., 17.5%) did not differ between treatments (χ^2 = 2.68, df = 3, p = 0.443; C 10.3%; F 14.3%; FA 20%; T 25.9%). Lastly, the number of different females mated with each male did not differ among treatments (range 1–6; see Cantarero et al. 2019 for statistics).

Implant treatments

All males received two subcutaneous implants (40 mm length, 1.47 mm i.d., 1.96 mm o.d.; Silastic tubing, Dow Corning, Midland, MI, USA) on the back. Males were randomly assigned to one of the four treatments. C-males (n = 30) received two empty implants. T-males (n = 29) received one of the implants filled with testosterone (Steraolids Inc.; Newport, RI, USA) plus the other empty one. F-males (n = 28) received an implant filled with F (Sigma-Aldrich, St. Louis, MO, USA) and another empty one. Finally, FA-males (n = 30) were treated with an implant filled with F and an implant filled with ATD (Steraloids Inc.). All implants were sealed at both ends with 1 mm of silicone glue (Nusil Technology, Carpinteria, CA, USA). All males were resampled for blood 25 days and 70 days after the implant.

The length of the testosterone implant was chosen on the basis of a previous study in red-legged partridges (Blas et al. 2006) and also studies from other bird species (see ESM and Cantarero et al. 2019 for further details). In the case of the antiandrogen-implants, the same length (40 mm) was chosen by predicting a proportional relation between the volume and effect of each compound. The plasma T levels of control, F-, FA-, and T-treated birds were, respectively (i.e., mean ± SD): 0.59 ± 0.71, 0.80 ± 0.09, 2.57 ± 1.05, and 4.50 ± 1.12 ng/mL when sampled 25 after the implant date of their first breeding season, and 0.48 ± 0.24, 0.58 ± 0.63, 1.34 ± 0.44, and 3.98 ± 1.00 ng/mL when sampled 70 days after that implant date (see

also ESM). Plasma T levels of T-treated males were significantly higher than those found in other groups (see Alonso-Alvarez et al. 2008 and Cantarero et al. 2019's data repository). The F-treatment did not significantly affect testosterone (see tests in Alonso-Alvarez et al. 2008) or estradiol (S1 Fig. in Cantarero et al. 2019) concentrations. In contrast, FA-implants led to intermediate (probably endogenous) testosterone concentrations, significantly higher than those in C-males but still lower than T-male concentrations (full description in Alonso-Alvarez et al. 2008). Higher T concentrations in FA-males compared to controls have always been reported in avian studies using FA-implants (ESM for literature). Lastly, estradiol concentrations only significantly and transitorily differed between FA- and C-males (i.e., 25 days after the implant date; see Cantarero et al. 2019, S1 Fig.), probably due to ATD-blocking activity being surpassed by endogenous T levels (see ESM).

Survival and reproduction monitoring

Survival was checked at least twice weekly during the 7-year period of this study. Egg-laying was monitored by removing the eggs from cages daily. Eggs were individually identified with a pencil, weighed (± 0.1 g) and stored at 15 °C. At this temperature, embryo development is arrested (Thear 1987). Stored eggs were transferred to incubators (37.7 °C) with an egg turning system (Massalles Mod. 65-I HLC, Barcelona, Spain). Five incubation batches (mean incubation period: 24 days) were programmed throughout each breeding season. The time in storage (0–21 days) before incubation did not differ among treatments and did not affect hatchability (details in ESM). Synchronizing hatching events was necessary to allow the management of large numbers of chicks in captivity (Alonso-Alvarez et al. 2010). The eggs were candled at 20 days from the incubation start by holding an intense light above or below the egg to detect embryo development. Those not showing a clearly visible air chamber generated by embryo gases were discarded. This procedure allowed us to manage the large numbers of eggs produced (N = 2535) and to accommodate viable eggs to the hatcher machine capacity (Massalles Mod. 65-N-HLC). Each egg in the hatcher was individually introduced into a small wire cage (10 × 5 × 5 cm) with an identification label, preventing hatchlings from mixing with other individuals and losing identification. Candling was not done in 2009–2011, because all the eggs could be introduced into the hatcher. The hatcher was reviewed daily. The hatchlings were identified using numbered rings (Velcro ©, Boston MA) on the tarsus, which were substituted by metallic rings at 14 days old. The Velcro rings were fixed with staples and reviewed every 2 days to avoid damages due to tarsus growth.

Lifetime hatching success was determined as the sum of all the hatchlings produced by the mate of a male divided by the sum of all the eggs produced by that bird. The number of embryos detected by candling divided by the number of eggs (embryo occurrence rate here and thereafter) was also tested, but separately for each year during the 2006–2008 period, i.e., when the data was available (see above). Embryo or hatching success variables were not calculated for males whose mates did not produce any egg during the analyzed period. The proportion of males whose mates did not produce eggs (25 from 114 birds, 21.9 %) was equally distributed among treatments (χ^2 = 0.70, df = 3, p = 0.995). Moreover, we calculated the overall chick survival probability using data of all chicks produced during the lifetime of a male by taking the total number of chicks that survived until 14 days old divided by the total number of hatchlings produced by the mate(s) of a male. All the chicks were weighed, and their size measured (tarsus length) at the hatching date and when they were 14 days old.

Effects on testis size

Seventeen males were experimentally treated and sacrificed in 2008 to test if the treatments affected testis size. These were a different subsample of yearlings (C-, F-, and FA-males: four birds per group; T-males: five birds). One F-male died 2 weeks after the implant date and was excluded from the analyses. Birds were housed and mated following the same procedure described above (same dates, housing and mating methods, etc.). Birds were sacrificed by cervical dislocation at the end of the breeding season. Testicles were immediately extracted, and their volume was calculated from the ellipsoid formulae, that is, volume = $4/3\ \pi\ ab^2$; a = 1/2 largest diameter; b = 1/2 minor diameter (Møller 1991). The total testicular volume was the sum of volumes of the left and right testicles.

Colibacillosis outbreak

The veterinary staff of IREC regularly monitored the sanitary status of the study population. An APEC (avian pathogenic *Escherichia coli*) enteritis outbreak was detected in December 2008 in our facilities. Culture and identification methods for APEC were performed as previously described (Blanco et al. 1997, 1998; Dho-Moulin and Morris Fairbrother 1999; Díaz-Sánchez et al. 2013). The first experimental birds died on December 12 and the last ones died on February 12, 2009. The number of birds of each experimental treatment that were alive just before the beginning of the outbreak did not differ between the two outdoor aviaries (aviary × treatment: χ^2 = 0.80, df = 3, p = 0.849), indicating that all the groups were equally exposed to a potential infection. The subsequent mortality due to the outbreak was virtually equal in both aviaries (aviary A: 11/26; aviary B: 12/26, 42% vs. 46% respectively, χ^2 = 0.78, df = 1, p = 0.780). Due to the persistence of the outbreak, all the birds housed in the two outdoor aviaries were treated with antibiotics and vitamins in drinking water

(Enrofloxacin 100/mg/mL; Floxaciven, Laboratorios Maimo, Barcelona). Twenty-three males died during this period.

Statistical analyses

Analyses were performed with IBM SPSS Statistics 24.0 (SPSS, Inc.) and SAS 9.4 version (SAS Institute Inc). Log-rank tests (Mantel-Cox) were used to analyze differences in survival of partridge males between treatments. Fourteen individuals died due to different accidents or their date of death could not be established precisely during the 7 years of the study (see ESM). The survivorship of these birds until their last record was considered as censored data. The sample sizes of censored vs. non-censored individuals were unbiased among treatments (χ^2 = 3.216, df = 3, p = 0.360). The total number of eggs, hatchlings or 14-day old survivor chicks, as well as embryo occurrence rate, total hatching success, and chick survivorship, showed distributions that could not be normalized. To overcome this limitation, non-parametric Kruskal-Wallis (K-W) and Mann-Whitney U tests were used for comparing groups. Instead, egg mass and offspring mass and size were normally distributed or normalized by log-transformation, being tested using one-way ANOVAs. The total testicular volume was analyzed using both non-parametric tests due to the lack of normality and low sample sizes. Finally, the survival of birds during the *E. coli* outbreak was tested by means of a chi-squared test.

Results

Survival

There was no effect of the hormonal treatment on survival when the four experimental groups were tested in the same model (log-rank test χ^2 = 2.329, df = 3, p = 0.127, Fig. 1). However, paired comparisons of the survival function indicated that mortality was significantly higher in T-males when compared to C-males (Table 1). Figure 1 shows a dramatic survival decline in T-males when the third year of life is surpassed. This coincides with the 2008–2009 colibacillosis outbreak described in "Methods" (see the arrow in Fig. 1).

The proportion of birds that died during the outbreak (December 12, 2008–February 12, 2009) differed among treatments (χ^2 = 8.227, df = 3, p = 0.042). Forty-two birds were still alive just before the outbreak (C 12, F 10, FA 10; T 10 birds). T-males showed 90% mortality during the outbreak period, whereas controls, F- and FA-birds reported 33%, 60%, and 40%, respectively. Pairwise comparisons showed that the survival of T-males was lower than that of C- and FA-males (both $p < 0.020$). The number of surviving males per experimental treatment just before the start of the outbreak did not differ between the two outdoor aviaries (χ^2 = 0.483, df = 3, p = 0.923), thus suggesting no link to a particular aviary. Moreover, survival tests performed just before the start of the outbreak also did not show significant differences among treatments (i.e., these log-rank tests assigned all the deaths registered after December 12, 2008, as censored data; χ^2 = 1.694, df = 3, p = 0.381). Similarly, pairwise comparisons between treatments just before the outbreak did not show differences among treatments (all log-rank tests $p > 0.20$).

Reproductive output

Table 2 shows the descriptive statistics of the lifetime reproductive output for each treatment group. The total number of eggs did not vary among treatments (K-W test, χ^2 = 0.408, p = 0.939). However, the total number of hatchlings and 14-day-old chicks, as well as lifetime hatching success, differed significantly among treatments (all K-W tests, $p < 0.01$; Table 2).

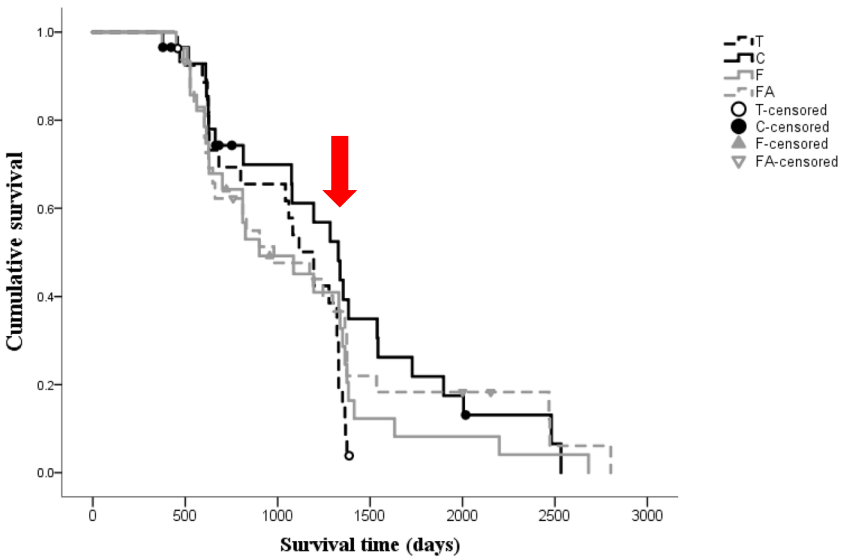

Fig. 1 Survival trajectories of partridge males exposed to hormone treatments. Control (C: solid black line), flutamide (F: solid grey line), flutamide + ATD (FA: dashed grey line), or testosterone (T: dashed black line) implants. The experiment started when birds were 1 year old. Note that the *E. coli* outbreak was brief regard to full lifespan in the population. It took place from 1321 to 1383 days (see also Methods). Dots on each line indicate censored data. The red arrow indicates the start of the *E. coli* outbreak

Table 1 Results of paired comparisons of survival distributions between hormonal treatment groups of red-legged partridges. Significant effects in italic. Subcutaneous implant group: *C*, control; *F*, flutamide (a blocker of androgen receptors); *FA*, flutamide plus ATD (an inhibitor of testosterone conversion to estrogens); *T*, testosterone

	F		FA		T	
Treatment	χ^2	*p*	χ^2	*p*	χ^2	*p*
C	1.326	0.249	0.349	0.555	*5.14*	*0.023*
F			0.494	0.482	0.834	0.361
FA					1.250	0.264

We found no difference among treatments neither in the mean mass of eggs, hatchlings, and 14-day-old chicks nor in their mean size (tarsus length) (all one-way ANOVA, $p > 0.15$). The lack of differences here indicates that male treatment did not affect female fecundity (i.e., the number of eggs laid) nor the quality of the offspring produced (i.e., total chick survivorship or egg and chick measures). Instead, results point to an effect on male fertility or embryo development. When pairwise comparisons were made for lifetime hatching success, T-males showed dramatically low hatching rates (Fig. 2; mean 16%) as compared to other groups (range of means 38–58%; all pairwise Mann-Whitney *U* tests, $p < 0.04$). Interestingly, F-males showed significantly higher lifetime hatching success than controls (U 146, $p = 0.038$) and similar to FA-birds (Mann-Whitney *U* test, $U = 217$, $p = 0.216$; see also Fig. 2).

When analyses are run for each year separately, the number of eggs produced by the mate(s) of a male did not differ among treatments (all years K-W tests, $p > 0.27$). However, the hatching success varied among experimental groups (see also S1 Fig. in ESM, including parametric statistics). The negative impact of the T-treatment on hatching success was more evident after the first implanting year (i.e., after 2006), whereas F- and FA-males showed higher hatching success than the other groups only during the first breeding season. Accordingly, the hatching success differed among groups in 2006 (K-W test, $\chi^2 = 16.15$, df = 3, $p = 0.001$), but not between T- and C-males (Mann-Whitney *U* test, $U = 12.05$, $p = 0.477$). F- and FA-males showed higher hatching success (mean: 56% and 47%, respectively) than controls (23%; Mann-Whitney *U* tests, $p = 0.013$ and 0.054) and T-males (9.3%; both $p < 0.014$). In 2007, the hatching success among groups showed a different pattern: the difference among treatments was again significant (K-W test, $\chi^2 = 11.00$, df = 3, $p = 0.012$), but here T- and C-males significantly differed (Mann-Whitney *U* test, $U = 49.5$, $p = 0.019$: means 12.5% and 49.4% respectively), whereas F- and FA-males (52% and 36.5%, respectively) did not differ from controls (both $p > 0.27$) but from T-males (both $p < 0.038$). The pattern as in 2007 was also found in 2008 (K-W test, $\chi^2 = 14.80$, df = 3, $p = 0.002$). Hatching success of T-males differed from other groups (all $p < 0.011$), but other groups did not differ among them (all $p > 0.21$). The three subsequent implanting breeding seasons (2009–2011) did not include T-males as none survived. In these latter years, no difference in hatching success among groups was detected (all K-W tests, $p > 0.40$; see also S1 Fig.).

Egg candling data

The experimental treatment also affected embryo occurrence rate during 2006–2008 (K-W tests: 2006: $\chi^2 = 30.5$, df = 3; 2007: $\chi^2 = 21.88$, df = 3; 2008: $\chi^2 = 25.69$, df = 3; all $p < 0.001$). Similar to hatching success, the T-treated group showed a lower embryo occurrence rate (range of means: 19–28%) than other groups (means always > 56%; all pairwise Mann-Whitney *U* tests, $p < 0.01$). Moreover, in 2006, F-males showed a higher embryo occurrence rate than controls (means: 87.5% vs. 56.6%, respectively; Mann-Whitney *U* test, $U = 43$, $p = 0.001$), FA-males showing a trend in the same direction (mean 72.4%; Mann-Whitney *U* test, $U = 101$, $p = 0.088$; other pairwise comparisons $p > 0.10$). The mortality between the candling and hatching date did not differ among treatments in any year (K-W tests, all $p > 0.180$).

Effect on testicular volume

Hormonal manipulations performed on the independent sample of birds sacrificed to assess testicular volume reported a significant effect of the treatment (K-W test, $\chi^2 = 9.11$, df = 3, $p = 0.028$). Pairwise comparisons showed that T-males showed a lower testicular volume than other groups (all Mann-Whitney *U* tests, $p < 0.037$; median and range in mm^3; C 131.5, 113.8–2045, $n = 4$; F 271.2, 92.0–382.7, $n = 3$; FA 339.9, 65.9–962, $n = 4$; T 36.4, 29.8–69.8, $n = 5$), whereas all other comparisons were non-significant (all $p > 0.68$).

Discussion

Our results show that the maintenance of high circulating levels of testosterone during every breeding season of life decreased survival probability after a colibacillosis outbreak. From a reproduction-survival trade-off perspective (Stearns 1992; Zera and Harshman 2001), the negative impact on fitness was not compensated by increased lifetime fecundity as the same birds showed a lower capacity to produce hatchlings. On the other hand, the antiandrogen treatment unexpectedly enhanced male reproductive success via increased egg hatchability. As far as we know, this is the first report of such a positive effect of flutamide in any species.

The impact of high testosterone levels on survival has been reported in at least four bird species, all of them manipulated

Table 2 Descriptive statistics of the reproductive output of male captive red-legged partridges during the entire lifetime. First five variables were calculated from total lifetime value, whereas the next five were calculated from mean values per male across its lifetime. Mass and tarsus length are reported in g and mm, respectively. See "Methods" for description of other variables

	Control (C)					Flutamide (F)					Flutamide + ATD (FA)					Testosterone (T)				
	N	Range	Mean	Median	SD	*N*	Range	Mean	Median	SD	*N*	Range	Mean	Median	SD	*N*	Range	Mean	Median	SD
Total number of eggs	29	0–117	23.00	13.00	30.20	28	0–78	21.14	17.00	21.10	30	0–103	23.97	17.00	25.50	27	0–58	20.63	17.00	18.70
Total number of hatchlings	29	0–69	12.14	1.00	19.02	28	0–61	11.64	7.50	14.70	30	0–42	10.30	6.50	11.63	27	0–19	2.07	0.00	4.28
Number of 14-day chicks	29	0–35	7.00	0.00	10.80	28	0–61	8.43	5.00	12.03	30	0–28	6.30	3.50	7.35	27	0–16	1.59	0.00	3.57
Total hatching success	21	0–84.61	37.82	36.54	30.27	22	0–100	57.84	60.43	24.06	25	0–100	45.60	50.00	32.35	21	0–100	16.16	2.56	27.69
Total chick survival	15	0–100	62.27	62.50	24.97	21	44–100	74.54	75.00	19.28	20	21.05–100	65.93	67.42	19.47	11	0–100	71.91	81.82	32.53
Mean egg mass	21	12.70–20.24	16.68	16.86	1.63	22	14.03–20.82	17.51	17.82	1.56	24	12.3–19.4	17.07	17.41	1.53	21	11.33–19	16.99	17.39	1.64
Mean hatchling mass	15	10.63–12.56	11.58	11.80	0.68	21	10.38–15.22	12.10	11.95	1.13	20	9.8–13.4	11.67	11.79	0.89	11	9.6–16.1	11.67	11.43	1.64
Mean hatchling tarsus length	15	14.35–16.26	15.25	15.06	0.64	21	13.93–17.77	15.46	15.23	1.06	20	12.99–16.0	15.07	15.28	0.82	11	13.9–19.6	15.65	15.38	1.43
Mean 14-day chick mass	14	25.88–87	47.80	45.85	17.61	21	26.22–96.95	62.15	63.70	19.81	20	27.3–94.65	58.41	59.50	18.70	10	23.8–79.5	55.49	61.00	20.99
Mean 14-day chick tarsus length	14	5.63–29.73	20.68	24.18	7.92	21	2.62–37.89	23.37	s26.43	8.41	20	2.73–37.4	24.07	25.63	6.91	10	23.8–30.2	27.56	28.20	2.10

under free-living conditions (Dufty 1989; Nolan et al. 1992; Moss et al. 1994; Saino et al. 1995). Captivity does not perfectly recreate natural scenarios and imposes limitations to evolutionary inferences (e.g., Ricklefs and Cadena 2007). However, it allows us to quantify longevity with absolute confidence and also to determine the cause of death more accurately. The experimental effects on survival did not indicate accelerated aging, being mostly due to a high mortality event when birds were more than 3 years old. This event was linked to an outbreak caused by a bacterial infection (i.e., extrinsic mortality). The outbreak took place during winter, that is, 5 months after removing the implants. We should remember that birds of all the groups were housed together during winter and, hence, should have been equally exposed to the infection.

Taking into account the well-known immune-competence handicap hypothesis (i.e., Folstad and Karter 1992), an intuitive explanation is that exogenous testosterone caused immunosuppression, making partridges more prone to disease. For instance, negative relationship between circulating T levels and the capacity of blood to kill *E. coli* bacteria was found in male red-winged blackbirds (*Agelaius phoenic*eus) (Merrill et al. 2015). Nonetheless, we should note that the implants were removed months before the outbreak. Removing T-implants just after the reproduction avoided winter mortality in wild male dark-eyed juncos (Nolan et al. 1992). In wild male red grouse, however, T-implants administered in autumn impaired body condition in the following spring, and even 1 year later, when the implants should be fully exhausted (Mougeot et al. 2005a; Martínez-Padilla et al. 2014b). Therefore, delayed immunosuppression could also be expected. Higher mortality in T-males could additionally be due to long-lasting effects on other condition components, such as the resistance to oxidative stress (Alonso-Alvarez et al. 2007), which is linked to immune response in birds (e.g., Costantini and Møller 2009). Testosterone effects on behavior could have also increased social interactions and pathogen transmission (e.g., Mougeot et al. 2005b; Escallón et al. 2017). However, the aviaries prevented to avoid social interactions, promoting a homogeneous rate of pathogen exposure.

The apparent impact of exogenous testosterone on fertility was not surprising considering avian studies reporting both increases and decreases in testicular size after a similar manipulation (review in Lofts and Murton 1973, and subsequent works such as Turek et al. 1976, 1980; Desjardin and Turek 1977; Hagen and Dziuk 1985; Kast et al. 1998; Deviche et al. 2006). It is assumed that testicular involution occurs via negative endocrine feedback (Turek et al. 1976; also Brown and Follett 1977; Desjardins and Turek 1977; Maung and Follett 1978; Kirby and Froman 2000; Fusani 2008; Vizcarra et al. 2015) but, interestingly this does apparently not arise if the androgen is provided when primary spermatozoa production is fully active (Lofts and Murton 1973 and references therein).

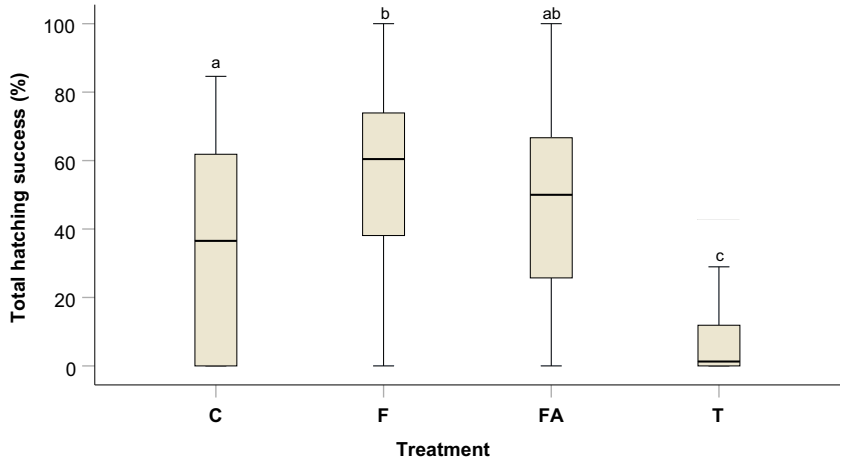

Fig. 2 Total hatching success (%) calculated as the number of hatchlings divided by the number of eggs produced by the mate of a male red-legged partridge during its lifetime. Males were annually treated with subcutaneous implants that were empty (C-males), filled with either a blocker of androgen receptors (flutamide; F), an inhibitor of testosterone conversion to estrogens (ATD) plus the cited blocker (FA) or testosterone alone (T). Box plot showing medians, quartiles, and ranges. Different letters over each bar indicate significant pairwise differences. See also "Results" and Table 2 for full descriptive statistics

Thus, captive male dark-eyed juncos T-implanted in mid-April did not show apparently significant effects on the ejaculate size extracted 2 months later (Kast et al. 1998). These dates coincide with our timing. We should note that partridges started to lay fecund eggs only a few days after the implanting date.

Although one could argue that our study involved non-natural testosterone levels, the concentrations of our T-males were well within the range reported for non-manipulated male red-legged partridges (Bottoni et al. 1993) and similar to levels in our C-males (Alonso-Alvarez et al. 2008). Interestingly, tiny testosterone dosages (1.5–2 mm long Silastic implants) reduced testicular mass in house sparrows *Passer domesticus* (Turek et al. 1976) even when not increasing circulating testosterone concentrations. This suggests that any exogenous testosterone dosage can impair male fertility. This is also supported by another experiment in male red-legged partridges where T-implants 10 mm shorter than here also induced a strong testicular involution (LP-R and J. Blas, unpublished data). Hence, the concerns raised by some authors regarding the negative influence of exogenous testosterone on male fertility (Foerster and Kempenaers 2004; Schwagmeyer et al. 2012; Martínez-Padilla et al. 2014a) are supported by our results. The negative impact of T-implants on male reproductive success underlines the inherent limitations of hormonal manipulations based on implants (Fusani 2008), which have, nonetheless, been used over decades (e.g., Wingfield 1984). An alternative approach is to test the individual capacity to increase testosterone quickly (in minutes) in response to a behavioral challenge (Wingfield 1990's "challenge hypothesis"), which should be mediated by a rise of gonadotropin-releasing hormone (GnRH) secretion (Nelson 2005). In dark-eyed juncos, GnRH injections raised LH and, consequently, T levels for ca. 30 min, but only those males generating intermediate T concentrations obtained higher fitness returns in terms of survival and fecundity (McGlothlin et al. 2008; McGlothlin et al. 2010). Other authors have followed this approach, but lifetime fitness effects were not addressed (e.g., Apfelbeck et al. 2011; Cain and Pryke 2017; Goymann and Flores Dávila 2017).

An intriguing result of our experiment was the increased lifetime hatching success (and also embryo occurrence) experienced by eggs laid by the mates of F-treated partridges, which was more evident in F-only treated males (i.e., F-males). Several potential explanations can be proposed: (1) higher investment in egg quality among females mated to F-treated males, (2) higher copulation rates in these groups, and/or (3) higher fertilization rates. In the first case, it is well known that female birds may manipulate the egg yolk composition (lipid content, immunoglobulins, or hormone levels; Groothuis and Schwabl 2008) depending on male attractiveness, and particularly, on carotenoid-based ornamentation (e.g., Giraudeau et al. 2011; Alonso-Alvarez et al. 2012). However, to accept this, we should first assume that F altered mate attractiveness, mothers accordingly inducing maternal effects that improved embryo viability, and hence, hatching success.

In the case of higher copulation rates among F-treated males, it is well-known that testosterone, not its blockage, promotes copulation (Nelson 2005). Moreover, F did not affect copulation rates in captive Japanese quails (*Coturnix japonica*) (Adkins-Regan and Garcia 1986; Alexandre and Balthazart 1986). Regarding increased fertility via sperm amount/quality, F leads to an impairment of spermatozoa production, either in quantity or quality, in mammalian models (Chandolia et al. 1991; Anahara et al. 2008; Lydka et al. 2011;

Hejmej and Bilinska 2018). Similar findings have been reported in frogs (Bhatia et al. 2014) and fishes (Orton et al. 2018). In birds (zebra finches *Taeniopygia guttata*), F subcutaneously implanted from 7 days old to adulthood, reduced testes size (Grisham et al. 2007). Nonetheless, dosages and time scales in all these studies differed with respect to our experiment. Contrarily, wild male rufous-collared sparrows (*Zonotrichia capensis*) treated with F plus ATD developed a larger cloacal protuberance than controls (Moore et al. 2004). The cloacal protuberance accumulates sperm, its volume being positively correlated to sperm quantity (Birkhead et al. 1993). Interestingly, in all avian studies, including our experiment, the FA-treatment (i.e., not F only) increase endogenous testosterone production (Moore et al. 2004 and ESM), which may influence sexual structures such as the cited protuberance. In any event, although FA also improved hatching success, the effect was more evident among partridges only treated with F, whose T levels did not differ from controls.

Despite this literature, if we assume that higher hatching success among F-treated males was indeed mediated by increased sperm quantity or quality, what mechanism could be involved? Studies in mammalian models suggest that F may induce compensatory processes increasing sensitivity to endogenous testosterone. Thus, F treatment in mice induced thyroid hormone receptor overexpression that, in turn, unblocked the androgen receptor activity (Liao et al. 2003). Moreover, long-term exposures of human cancer cells to F can stimulate, instead of blocking, the androgen receptors (Miyamoto et al. 1998) or increase the production of these androgen receptors (Chen et al. 2003). The effect on fecundity suggests that F-treated birds should have similarly increased the number of receptors to endogenous testosterone, particularly those in Sertoli cells in charge of spermatogenesis, which have been described in birds (e.g., Leska et al. 2012; Kiezun et al. 2015). Therefore, we can tentatively suggest that a compensatory receptor-based mechanism could have improved sperm production and, consequently, lifetime hatching success in our F-male partridges.

In summary, our results as a whole suggest that those particular phenotypes maintaining high circulating levels of testosterone during the breeding season throughout their life would be negatively selected as a consequence of their predisposition to death by infections, which agrees with most accepted theory (Folstad and Karter 1992; Hau 2007). We should, nonetheless, recognize that the effect in the wild could be weaker as captivity promotes high rates of infection and transmission of pathogens. In fact, it has been shown that the prevalence of APEC infection among wild red-legged partridges is lower than that among captive non-manipulated individuals (3% vs. 11% respectively, Díaz-Sánchez et al. 2012). We should also consider that the decline in hatching success among T-males might not be the result of the reproductive vs. longevity trade-off, but a consequence of the experimental manipulation. On the other hand, antiandrogen treatment did not improve survival at the expense of reproduction but instead improved reproductive success via hatching success. If this was a consequence of a compensatory increase in the amount/activity of gonad androgen receptors (see above), we may infer that such an amount/activity could be constrained under natural conditions. To explain why such a hypothetical constraint could evolve is, however, difficult as it should theoretically rely on costs derived from androgen receptor overexpression, which are unknown. More experimental studies are, therefore, needed to test all these ideas and to clarify the role of androgen-receptor expression on breeding success and, hence, on fitness.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This investigation was approved by the institutional animal welfare committee (University of Castilla-La Mancha's Committee on Ethics and Animal Experimentation) in accordance with pertinent Spanish (RD1201/2005) and European Union (86/609/CEE and 2010/63/EU) legislation under Protocol number 1011.01. The implanting procedure was performed under veterinary supervision. We used a planned humane endpoint where any bird would be immediately euthanized by cervical dislocation when it rapidly lost more than 20% of body mass.

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