

Testosterone-mediated trade-offs in the old age: a new approach to the immunocompetence handicap and carotenoid-based sexual signalling

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The immunocompetence handicap hypothesis proposes that testosterone mediates a trade-off between sexual signalling and immunocompetence in males. Such a trade-off could favour the reliability of sexual signals on the basis that testosterone required for signal expression also promotes immunosuppression. However, the immunosuppressive activity of testosterone has not been convincingly demonstrated. We propose that the optimal solution to the testosterone-mediated trade-off should change with age, explaining ambiguous results in the past. Testosterone and ageing would promote two simultaneous immunosuppressive challenges unaffordable for low-quality males. Oxidative stress, as intimately related to ageing and immunosenescence, could contribute to enhance signal reliability. In this context, traits coloured by carotenoids (yellow–red traits) could play a crucial role due to the immunostimulatory and antioxidant properties of these pigments. Here, old and middle-aged male red-legged partridges were treated with testosterone or manipulated as controls. In the presence of high-testosterone levels, middle-aged males increased both circulating carotenoid levels and colour expression, whereas their cell-mediated immunity was not significantly altered. However, in old males, neither circulating carotenoids nor sexual signalling increased when treated with testosterone, but immunosuppression was detected. The link between testosterone and carotenoids could favour the reliability of sexual signals throughout the life.

Keywords: antioxidants; evolutionary trade-offs; sexual secondary traits; sexual selection; senescence; oxidative stress

1. INTRODUCTION

Current sexual selection theory postulates that sexual secondary traits may act as honest (i.e. reliable) signals of the phenotypic/genotypic quality of individuals (Andersson 1994). That reliability has been linked to the fact that resources allocated to signalling should trade off against survival or viability. High-quality signallers would be those more efficient in managing the trade-off (Getty 2006). The immunocompetence handicap hypothesis (ICHH) proposes that testosterone mediates a particular trade-off between sexual signalling and immunocompetence in males (Folstad & Karter 1992). Such a trade-off would be negative at the individual level, assuming that high blood levels of testosterone, required for the full expression of sexual signals, also increase the risk of mortality due to the immunosuppressive action of this hormone. In other words, only males that are able to afford the suppression of the immune system would be able to maintain sexual signal expression at high levels (Folstad & Karter 1992).

Since the ICHH publication (1992), much effort has been made to demonstrate the immunosuppressive properties of testosterone, but the results have not been conclusive (reviewed in Roberts *et al.* 2004). Many mechanistic alternatives have been proposed to explain

the inconsistency of the results in the literature, suggesting the presence of confounding variables or intermediate physiological pathways that would mask the impact of testosterone on immunity (Owen-Ashley *et al.* 2004; Blas *et al.* 2006; Alonso-Alvarez *et al.* 2007a,b; McGraw & Ardia 2007; Kempenaers *et al.* 2008).

On the other hand, since current signalling theory predicts that high-quality big signallers should pay proportionally lower marginal costs for signalling (Getty 2002, 2006), immunosuppression could be difficult to detect in some individuals. High-quality individuals exposed to high testosterone levels could better afford the cost, only revealing a moderate immunosuppression. Thus, a sample restricted to high-quality individuals would prevent to detect the impact of testosterone on the immune system. To take into account this possibility, a good knowledge of the individuals' life history is necessary. Here, a decisive question is how the age affects the solution of the trade-off. A trend towards interpreting sexual signalling from a life-history perspective has gained relevance during the last decades (Nur & Hasson 1984; Iwasa *et al.* 1991; Kokko 1997; Proulx *et al.* 2002; Getty 2006). However, the role played by age in the solution of the testosterone-mediated trade-off, as far as we know, has never been experimentally addressed.

Life-history theory predicts that individuals from iteroparous species should increase reproductive investment

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as they become older because their future breeding opportunities fade (i.e. assuming all else being equal; Stearns 1992). Among the investments required for reproduction, sexual signalling is decisive because the better the signal, the higher the mating chance, and ultimately, the better the reproductive outcome (Andersson 1994). Some theoretical models aiming to explain male sexual signalling predict not only an increase in the signal intensity with age (Kokko 1997), but also an increase in signal reliability (Proulx *et al.* 2002). The latter owing to the fact that old males should always maximize their reproductive investment—including signalling—relative to younger males, and hence, their signals should fit better to their own individual quality (Proulx *et al.* 2002). From this point of view, females assessing old males as potential mates would be less likely to be wrong. Several correlational studies in vertebrates give support to these predictions (Candolin 2000; Poesel *et al.* 2006; Judge *et al.* 2008; but see Badyaev & Duckworth 2003). However, it is unknown what kind of proximate mechanisms may favour an increase in signal reliability with age. The study of the ICHH within the framework of age-related physiological changes may offer a new perspective.

In old males, the immunosuppressive activity of testosterone would be merged with the immunodepression caused by damage and physiological constraints associated with ageing (i.e. immunosenescence). At the basis of immunosenescence is oxidative stress. Oxidative stress is the result of the imbalance between the production of reactive oxygen species (ROS) by normal metabolic activity and the state of the repair and antioxidant machineries (Finkel & Holbrook 2000). It is broadly assumed that the accumulation of damages produced by ROS throughout life is the main proximate cause of the ageing process (Finkel & Holbrook 2000). Furthermore, oxidative stress impairs cell-mediated immunity in vertebrates (Haussmann *et al.* 2005), leading to an age-related decline in the capacity to mount an efficient immune response (Larbi *et al.* 2007). Interestingly, testosterone seems to promote oxidative stress (Alonso-Alvarez *et al.* 2007b; Kurtz *et al.* 2007), probably due to increased metabolic rates (e.g. Buchanan *et al.* 2001), which ultimately lead to higher ROS production (Finkel & Holbrook 2000). In this sense, the ICHH has been recently refined as the ‘oxidation handicap hypothesis’, suggesting that only high-quality males should be able to afford the oxidative challenge induced by testosterone (Alonso-Alvarez *et al.* 2007b).

We hypothesize that the solution to the testosterone-mediated trade-off between signalling and immunocompetence depends on the individual’s age. We predict that, in the presence of high testosterone levels (i), older males should invest preferentially in signalling due to their lower residual reproductive value (see ‘terminal investment hypothesis’; e.g. Velando *et al.* 2006), or alternatively (ii) older males are unable to signal due to the accumulation of damages throughout life (i.e. a constraint; Kirkwood & Austad 2000; Torres & Velando 2007). In both cases, we predict that the immunosuppressive effect of testosterone assumed by the ICHH would be stronger in older males due to immunosenescence, assuming that the immune system of old males would be *a priori* debilitated.

Coloured traits produced by carotenoid pigments (yellow–red traits) are particularly good candidates to act

as reliable signals in this context. Carotenoid-based sexual signals are present in different taxa and are well described in some bird species (Hill & McGraw 2006). Carotenoids are antioxidant and immuno-stimulatory compounds that are only acquired with food (i.e. not synthesized by the organism; Chew & Park 2004; Palozza *et al.* 2006). Thus, individuals should carefully optimize carotenoid allocation between competing functions: expression of coloured signals; immune response; and/or combat against oxidative stress (Lozano 1994; von Schantz *et al.* 1999). Interestingly, it has been recently shown that male birds treated with exogenous testosterone increase their levels of circulating carotenoids (Blas *et al.* 2006; McGraw *et al.* 2006; Alonso-Alvarez *et al.* 2008), perhaps to buffer the testosterone-induced immunosuppression and/or oxidative stress (Blas *et al.* 2006; McGraw & Ardia 2007; Alonso-Alvarez *et al.* 2008).

To test our hypothesis and predictions, captive middle-aged and old male red-legged partridges (*Alectoris rufa*) were subcutaneously implanted with testosterone (T-males) or they received an empty implant (C-males). In this species, the red integument around the eyes is a sexually dimorphic trait coloured by carotenoids (Pérez-Rodríguez 2008). The red pigmentation is positively correlated with cell-mediated immune response and overall body condition (Pérez-Rodríguez & Viñuela 2008; Pérez-Rodríguez *et al.* 2008), and females increased their reproductive investment (i.e. clutch size) when the redness of their mates was experimentally intensified (C. Alonso-Alvarez 2008, unpublished data). Carotenoid-based coloration, plasma carotenoid levels, plasma antioxidant status and level of lipid peroxidation in erythrocytes (a proxy of oxidative damage in cell membranes) were assessed before and after the treatment. The effect of the interaction between age and treatment was tested. To assess immunocompetence, cell-mediated immunity (CMI) was measured once at the end of the experiment.

2. MATERIAL AND METHODS

(a) *Experimental procedure*

The study was carried out on 68 captive male partridges housed in a governmental breeding facility at Chinchilla (Albacete, NE Spain). This facility maintains a large captive population obtained from free-living chicks, which were captured in a close non-managed area (15 000 ha owned by the Spanish army). Adult birds are housed in couples in outdoor cages (1.2 × 0.8 × 0.6 m), being fed *ad libitum* with commercial pelleted food (Nanta foods, Spain). Birds are annually marked with coloured rings indicating the year of birth. Two age-classes were used for the study: 2-year-old birds and birds more than 5 years of age. Birds in the first category ($n=34$) were chosen because they are sexually mature adult birds (maturity is attained 1 year earlier in this species), presenting higher breeding investment and productivity than yearlings (Cabezas-Díaz *et al.* 2005). Old birds were 6 ($n=18$), 7 ($n=13$) and 8 ($n=3$) years old. Red-legged partridges older than 5 years can be considered as senescent birds since they produce fewer offspring (reproductive senescence) and present higher levels of oxidative damage than younger birds (i.e. lipid peroxidation; C. Alonso-Alvarez 2008, unpublished data). The number of partridges assigned to each treatment was balanced among age classes ($\chi^2_4=0.41$, $p=0.98$).

Blood samples were taken on 11 April and 5 May, 2007 (pre- and post-treatment samples, respectively). Samples were obtained within 2 min after the removal of a bird from its cage, were immediately stored at 4°C until centrifugation at 7200g and plasma extraction, which was done within 10 hours. Both plasma and cell fraction (pellet) were frozen at -80°C until analysis. The birds were surgically implanted on 18 April. Digital images of the head were taken on this date, and again on 5 May. An immune challenge was performed on all the birds on 15 May. These dates coincided with the mid-point of the breeding season and the period of maximum ornament expression in captive males of this species (Pérez-Rodríguez 2008).

All males received a subcutaneous implant (30 mm length, 1.47 mm i.d., 1.96 mm o.d.; Silastic tubing, Dow Corning, Midland, MI). T-males (i.e. $n=34$) received an implant filled with testosterone (Steraolids Inc., NewPort, RI) and C-males ($n=34$) received an empty implant. Implants were sealed at both ends with 1 mm of silicon glue (Nusil Technology, Carpinteria, CA). The length of the implant was established on the basis of previous work on this species (Blas *et al.* 2006; Alonso-Alvarez *et al.* 2008). To avoid unnecessary suffering of the birds we followed protocols in concert with Spanish laws and the veterinarian staff of the Instituto de Investigación en Recursos Cinegéticos.

(b) Testosterone assays

Plasma testosterone concentration was determined using an enzyme immunoassay kit from DRG International, Inc. (Mountainside, NJ). These kits have been successfully used in quails showing recovery rates greater than 95 per cent (Wilhelms *et al.* 2005). Sensitivity is established at 0.083 ng ml⁻¹. Here, repeatability (i.e. Lessells & Boag 1987 and thereafter) calculated from a subset of samples ($n=30$) measured twice on the same session (intra-assay repeatability) or in different sessions (inter-assay repeatability) was high ($r=0.95$ and 0.90 , respectively). Samples from the same bird were assessed in the same session. Treatment and age groups were randomly distributed among plates.

(c) Cell-mediated immune response

The phytohaemagglutinin injection assay was used to evaluate the immune response mediated by T lymphocytes. Nonetheless, the specificity of this technique is currently under debate (see Martin *et al.* 2006; Tella *et al.* 2008). Thus, the broad term cell-mediated immunity (CMI) was used to indicate any response mediated by immune cells (adaptive or innate). The birds were injected subcutaneously in the wing web with 100 µl of 5 mg ml⁻¹ PHA (phytohaemagglutinin Sigma, St Louis, MO) diluted in PBS (phosphate buffered saline). The thickness of the wing web was measured at the injection site just prior to and 24 hours after the challenge. A spessimeter was used (± 0.01 mm; Mitutoyo Absolute 547-315, Japan). Repeatability of our measurements taken was high ($r=0.95$) and thus mean values were used in the analyses. The wing-web swelling (CMI) was calculated as the difference between the mean thickness prior to and 24 hours after injection.

(d) Plasma antioxidants

Plasma antioxidant status was assessed by using commercial kits (Randox Laboratories, Crumlin, UK) adapted to an automated spectrophotometer (A25-Autoanalyzer, Biosystems, Barcelona). The method is based on Miller *et al.*'s (1993) protocol. Plasma samples were incubated

during 15 s with a chromogen composed of metmyoglobin and ((2,2-azino-di-[3-ethylbenzthiazoline sulphonate])); ABTS). Then, hydrogen peroxide (H₂O₂) was added and the sample was incubated for 195 s. H₂O₂ addition induces the production of the radical cation ABTS, which generates a blue-green colour. Colour is measured at 600 nm before and after H₂O₂ addition, determining the colour change. Antioxidants in plasma cause suppression of this colour to a degree that is proportional to their concentration. Results are given as mmol l⁻¹ of total antioxidants in plasma. No value reached the linearity limit (i.e. 2.5 mmol l⁻¹) or the minimum detectable value (0.5 mmol l⁻¹; Randox laboratories). Repeatability was confirmed using a random subset of samples measured twice ($r=0.94$, $p<0.001$, $n=20$).

Cohen *et al.* (2007) have recently suggested that this measure must be corrected in birds by uric acid concentration in plasma. Thus, the concentration of this metabolite was also assessed and the change in their plasma levels was included as a covariate in the model testing the change in antioxidant status (table 1, note below). Plasma uric acid values were determined by spectrophotometry and commercial kits (Biosystems SA, Barcelona), following the uricase/peroxidase method. According to Cohen *et al.*'s (2007) results and previous work in partridges (Pérez-Rodríguez *et al.* 2008), the change in uric acid level was positively related to the change in antioxidant status (Pearson's $r=+0.57$, $p<0.01$).

(e) Oxidative damage in erythrocytes

Lipid peroxidation was assessed following Aust's procedure (Aust 1985). The principle is based on the fact that most tissues contain a mixture of thiobarbituric acid reactive substances, including lipid hydroperoxides and aldehydes, the concentrations of which increase due to oxidative stress. Briefly, the blood pellet was thawed and immediately diluted (1 : 10) and homogenized in a stock buffer (0.01 M PBS and 0.02 M EDTA (ethylenediaminetetraacetic acid)), working on ice to avoid oxidation. One millilitre of the homogenate was mixed with 2 ml of a solution (trichloroacetic acid 15%, HCl at 0.25 N and thiobarbituric acid 0.375% all in H₂O) and with 20 µl of diluted BHT ((Butylated hydroxytoluene) at 2% in ethanol), all in closed glass tubes. The tubes were then warmed for 30 min at 90°C and afterwards cooled with ice-cold water (10 min). The supernatant absorbance was measured by spectrophotometry at 535 nm after centrifugation (2025g, 15 min). Concentrations of peroxidized lipids were determined by comparing absorbances with those obtained from a curve with 0, 1.25, 2.50 and 5 nmol ml⁻¹ of malondialdehyde (MDA) in H₂O (i.e. end product of lipid peroxidation; Aust 1985). Concentrations of peroxidized lipids per gram of pellet are expressed in units of MDA in H₂O (i.e. end product of lipid peroxidation). Repeatability was estimated on a subset of samples ($r=0.80$, $p=0.03$, $n=20$).

(f) Carotenoids

Total plasma carotenoid concentration was determined by spectrophotometry, using a standard curve of lutein (Sigma, St Louis, MO). Plasma samples (60 µl) were diluted in acetone (1 : 10) and mixed, and the flocculent protein was precipitated by centrifuging at 11 000g for 10 min. The supernatant was examined in a spectrophotometer (Shimadzu UV-1603, Japan) and the absorbance was determined at 446 nm. Carotenoid values assessed twice on a subsample were highly repeatable ($r=0.99$, $p<0.001$, $n=20$).

Table 1. General linear models analysing the influence of the age class and the testosterone treatment on the change (post- minus pre-treatment values) of several dependent variables, including the pre-treatment value as a covariate. (η^2 : proportion of explained variance (see §2*h*). Italics indicate significant *p*-values.)

	pre-treatment value				age				treatment				age × treatment			
	F	d.f.	p-value	η^2	F	d.f.	p-value	η^2	F	d.f.	p-value	η^2	F	d.f.	p-value	η^2
plasma testosterone	98.58	1,63	<0.001	39.8	0.08	1,63	0.887	0.01	85.39	1,63	<0.001	34.5	0.423	1,63	0.518	0.2
body mass	0.016	1,63	0.899	0.02	0.78	1,63	0.380	1.2	4.05	1,63	0.049	6.1	0.443	1,63	0.508	0.7
T-cell-mediated immunity					1.25	1,64	0.269	1.8	0.971	1,64	0.328	1.4	6.87	1,64	0.011	9.8
antioxidant status ^a	35.76	1,62	<0.001	22.0	2.25	1,62	0.139	1.3	4.75	1,62	0.033	3.2	2.71	1,62	0.105	1.8
lipid peroxidation	41.1	1,63	<0.001	36.7	2.69	1,63	0.106	2.4	0.27	1,63	0.762	0.5	0.27	1,63	0.765	0.5
plasma total carotenoids	17.86	1,63	<0.001	21.7	1.56	1,63	0.217	1.8	1.80	1,63	0.185	1.9	5.54	1,63	0.022	7.6
proportion of red area	22.82	1,63	<0.001	25.8	1.46	1,63	0.231	1.7	1.75	1,63	0.192	2.1	5.71	1,63	0.020	6.6
red intensity	63.18	1,63	<0.001	48.3	10.92	1,63	0.002	8.3	0.515	1,63	0.476	0.4	4.14	1,63	0.047	3.2

^aModel testing the change in antioxidant status also included the change in plasma uric acid levels as a covariate ($F_{1,62} = 11.69, p < 0.001, \eta^2 = 10$; see §2).

(g) Colour assessment

Digital images of the head were taken at each sampling event under standardized light conditions, using a standard grey chip (Kodak, New York City, NY) placed close to the bird. Colour intensity was measured on pictures using ADOBE PHOTOSHOP v. 7.0. Analyses were performed by the same person who was blind to the bird's identity. The eye ring of the red-legged partridge shows a striking degree of variation in the amount of bare skin around the eye pigmented by carotenoids or unpigmented (i.e. showing the white-underlying dermis). The proportion of pigmented area on the bare lore and eye ring ('proportion of red surface' hereafter) was determined by selecting the red surface, dividing the number of red (carotenoid-pigmented) pixels by the total number of pixels of the area. The redness of the pigmented area was also determined by recording mean values of red, green and blue components (RGB system; [Blas et al. 2006](#)). Mean RGB values obtained per duplicate were repeatable ($r=0.91$, $p<0.001$, $n=68$), average values being used. Hue was determined after conversion of RGB values by ADOBE software. High values of hue denote less redness. To facilitate the interpretation, the sign of the hue value was reversed in the analyses and figures and this new variable was termed as 'red intensity'.

(h) *Statistical analyses*

Two-way ANOVAs were used to test differences between age-classes and treatments on the pre-treatment values. Afterwards, general linear models were carried out to test both experimental and age-related effects. The change (i.e. pre-treatment minus post-treatment value) was included as a dependent variable. Age-class and treatment were included in the model as fixed factors, testing also its interaction. The pre-treatment value was also included as covariate. In models testing colour, the hue of the reference chip was tested as a covariate to control for potential subtle changes in lighting conditions. However, its effect was never significant (all p 's > 0.15), being removed from the models. Similarly, several covariates were tested to understand the relationship between different parameters, being removed when non-significant ($p > 0.05$). The significance of independent factors did not change when non-significant interactions were removed from the saturated model (table 1). The homocedasticity requirement was always met (Levene F -tests). Testosterone levels were log transformed to normalize data. Means $\pm$ s.e. are showed. Effect sizes are presented as eta-square (η^2) values (i.e. ratio of the sum of squares of each term to total sum of squares of the model; table 1).

3. RESULTS

At the start of the study, old males presented less red intensity (i.e. higher hue) than middle-aged males (pretreatment hue values: $18.79^{\circ} \pm 0.50^{\circ}$ and $16.8^{\circ} \pm 0.49^{\circ}$, respectively; $F_{1,66} = 5.85$, $p = 0.018$). At that time, old males also presented lower plasma carotenoid levels than middle-aged birds (means $\pm$ s.e.: 4.06 ± 0.39 and $4.93 \pm 0.46 \mu\text{g ml}^{-1}$, respectively; $F_{1,66} = 4.37$, $p = 0.040$). Pretreatment testosterone levels were higher in old males (old birds: $3.56 \pm 1.75 \text{ ng ml}^{-1}$; middle-aged birds: $1.69 \pm 0.35 \text{ ng ml}^{-1}$), but this difference was only marginally significant ($F_{1,66} = 3.37$, $p = 0.071$). No other variable showed significant differences between age classes and treatments at the start of the study (including the interaction: always p 's > 0.09).

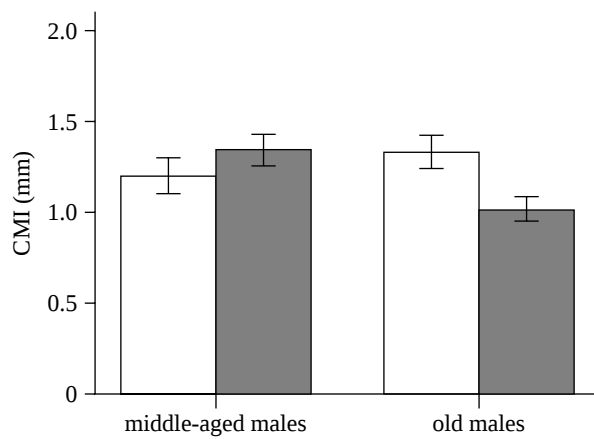


Figure 1. Cell-mediated immunity (CMI) response in middle-aged and old male partridges treated with testosterone (filled bars) or manipulated as controls (open bars). Error bars represent means $\pm$ s.e.m.

Afterwards, as expected, T-males showed the highest testosterone concentration (means $\pm$ s.e., range; T-males: 5.75 ± 0.39 , 3.51 – 11.86 ng ml $^{-1}$; C-males: 1.07 ± 0.36 , 0.08 – 8.77 ng ml $^{-1}$). T-males showed testosterone levels similar to those detected in untreated males of this species (Bottoni *et al.* 1993; Alonso-Alvarez *et al.* 2008). The observed change in testosterone levels was exclusively due to the treatment (table 1).

Similarly, treatment was the only factor significantly affecting body mass change (table 1). T-males gained body mass throughout the experiment, whereas C-males showed the opposite pattern ($+5.74 \pm 3.2$ g and -4.56 ± 3.95 g, respectively). Since changes in body condition or food intake could bias results, variation in body mass was included as a covariate in subsequent models, being removed however as non-significant term in all cases (all $ps > 0.30$).

In the model testing CMI, the treatment $\times$ age interaction was significant (table 1, figure 1). Immunosuppression mediated by testosterone was evident in old males (least squared difference (LSD) *post hoc*: $p = 0.015$), whereas in middle-aged males the effect seems to be the opposite, although differences between treatments were not significant (LSD *post hoc*: $p = 0.241$).

The testosterone treatment, but not the age, influenced the change in plasma antioxidant status (table 1), T-males showed reduced values with respect to control males (figure 2a). To test if carotenoids acted as antioxidants, carotenoid change was also added as a covariate in the same model. It explained part of the variability in antioxidant status ($F_{1,61} = 5.76$, $p = 0.02$, $\eta^2 = 3.7\%$, $r = +0.28$), but this did not modify the effect of the treatment ($p = 0.013$). With regard to lipid peroxidation, only age showed a trend to significance in the saturated model (table 1). However, when non-significant terms were removed, the age became significant, old males showing higher lipid peroxidation than middle-aged males ($F_{1,66} = 3.02$, $p = 0.039$, $\eta^2 = 5.6\%$; pre-treatment values: $F_{1,66} = 30.01$, $p < 0.001$; $\eta^2 = 39.2\%$; figure 2b).

With regard to colour variables, the interaction treatment $\times$ age showed a significant effect on the change in the proportion of red surface (table 1). Only middle-aged T-males showed a clear rise (figure 3a).

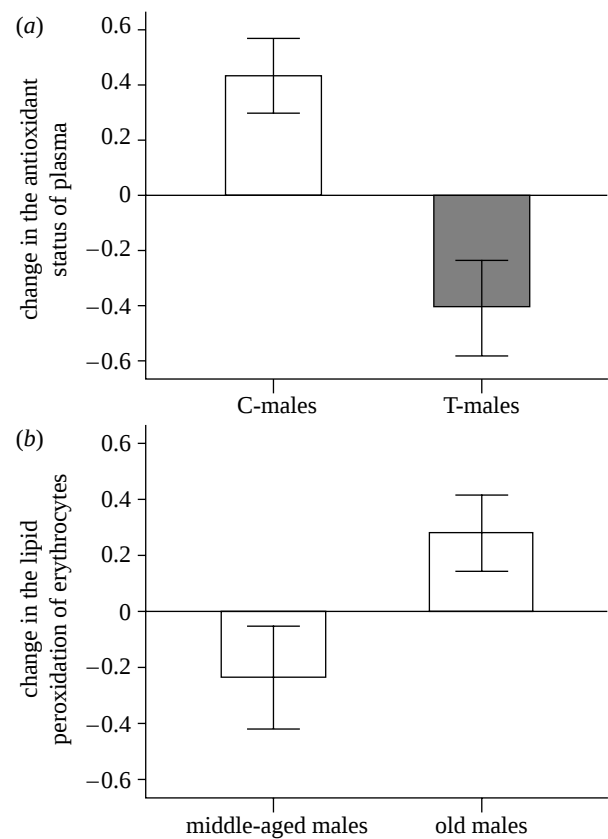


Figure 2. Change in (a) the antioxidant status of plasma in male partridges treated with testosterone (filled bars) or manipulated as controls (open bars), and change in (b) the lipid peroxidation of erythrocytes partridges of different ages. Values are residuals from models in table 1. Error bars represent means $\pm$ s.e.m.

A similar result was obtained for the change in red intensity (table 1; figure 3b), in this case old T-males showing a sharp decline in redness (figure 3b).

Finally, the change in carotenoid levels differed between treatments and age classes (see interaction in table 1). Only middle-aged T-males showed a clear increase in the amount of circulating carotenoids (figure 3c).

To look deeper into the physiological mechanisms that could explain our findings, we further tested the potential influence of correlation between parameters. Treatment $\times$ age interaction in the model testing the change in red intensity (table 1) vanished (both p 's > 0.10) when either the pre-treatment carotenoid level or the change in carotenoid values was separately added as covariates (both $ps < 0.020$). This suggests that variability in blood carotenoid levels explained the loss of redness in old T-males. Both covariates were positively correlated to the change in red intensity (Pearson's $r = +0.41$, $p = 0.010$ and $r = +0.25$, $p = 0.048$, respectively), that is, the higher the initial carotenoid level and/or the larger the carotenoid increase, the higher the rise in red intensity. CMI variability was also influenced by the change in carotenoid levels (covariate: $F_{1,62} = 4.45$, $p = 0.039$, $r = +0.23$), which removed the significance of the interaction ($F_{1,62} = 1.80$, $p = 0.185$), suggesting that the immunosuppressive effect of testosterone was due to an incapacity to increase the amount of circulating carotenoids in old males. No other parameter was correlated with CMI or colour change (all $ps > 0.20$).

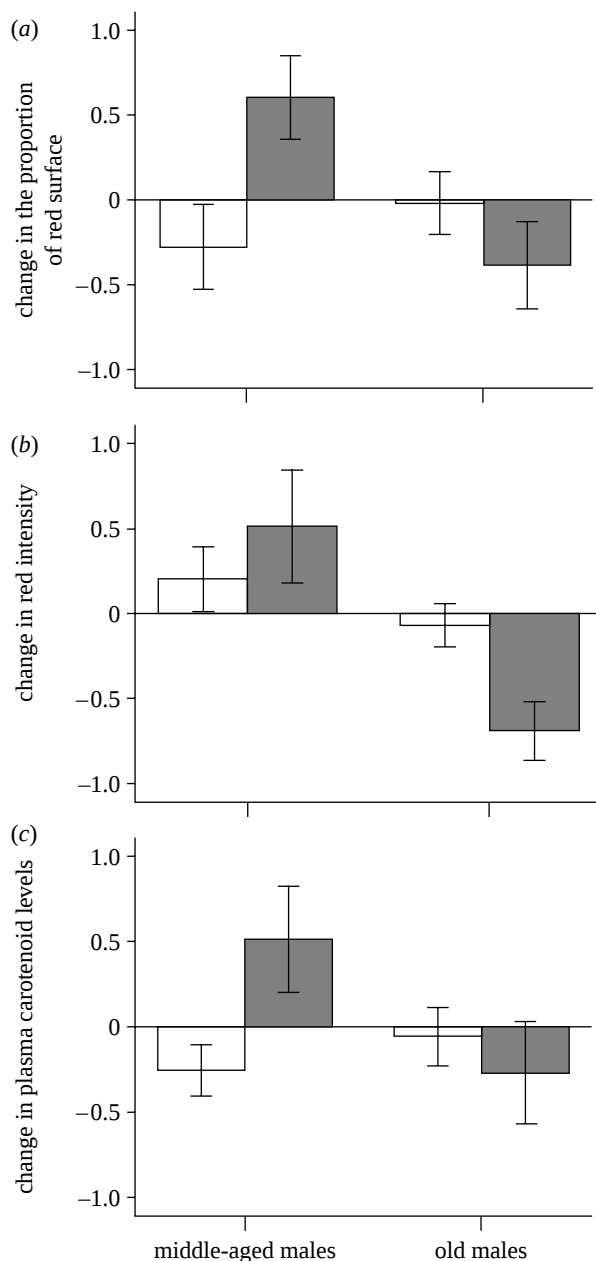


Figure 3. Change in (a) the proportion of red surface around the eyes, (b) the red intensity of the cited red surface and (c) the level of plasma carotenoids in middle-aged and old male partridges treated with testosterone (filled bars) or manipulated as controls (open bars). Values are residuals from models in table 1. Error bars represent means $\pm$ s.e.m.

4. DISCUSSION

Our results demonstrate the immunosuppressive action of testosterone, but only in old males. Old males also showed paler traits than middle-aged males before manipulation, and did not increase the expression of sexual signal when exposed to high testosterone levels. Our findings support the hypothesis that the solution to the testosterone-mediated trade-off between signalling and immunocompetence depends on the individual's age. They also indicate that red traits in partridges signal immunocompetence and probably, the capacity to efficiently manage carotenoids as a limiting resource in the trade-off. The latter seems to support the prediction of a physiological constraint probably due to the accumulation of damages throughout life (Finkel & Holbrook 2000). These findings underline the

importance of considering the age of individuals when testing the ICHH. The omission of such a key life-history parameter could explain inconsistent results reported in the past (review in Roberts *et al.* 2004).

At a first glance, our results provide a potential mechanism allowing an increase in signal reliability with age, as predicted by some theoretical models (Kokko 1997; Proulx *et al.* 2002). High testosterone levels and ageing would induce two simultaneous costs unaffordable for low-quality males. However, taking into account that middle-aged males did not show any detectable cost in terms of immunosuppression, a crucial question arises about how a correlation between signal expression and individual quality, allowing signal reliability, can be stabilized in the population. The answer could be that the red trait essentially signals phenotypic and/or genotypic quality, and that this quality may correlate with age. Here, middle-aged birds could have been birds of higher quality, able to invest resources in both branches of the trade-off (i.e. signalling and immunocompetence). In contrast to the apparent high quality shown by middle-aged males, early theoretical models predicted that females should prefer older males as mates because, merely by surviving, they have proven their high genotypic quality for viability (Manning 1985; Brooks & Kemp 2001). Older males could also provide more resources to reproduction or reduce the costs of mating and breeding due to their experience (Brooks & Kemp 2001). However, older males may present poor body condition, immunodeficiency (exposing females to contagious infections), and lastly, infertility (Brooks & Kemp 2001). They could also carry genes less adapted to current environment conditions, accumulate deleterious germ-line mutations or carry genes that, by maximizing early-age breeding, minimize old-age reproductive capacity (Brooks & Kemp 2001; Radwan 2003; Pizzari *et al.* 2007). In this line, recent theoretical and experimental studies support females' preference for middle-aged males (Jones & Elgar 2004; Beck & Promislow 2007).

In such an age-related signalling context, carotenoid-based traits could be good candidates to play the role of reliable quality signals throughout the life. Torres & Velando (2007) have recently shown how an experimental inflammatory challenge promoting lipid peroxidation is able to inhibit a carotenoid-dependent signal in male blue-footed boobies (*Sula nebouxi*), the effect being stronger in old males. As far as we know, Torres and Velando's study is the first to show an age-related decrease in the expression of a carotenoid-based sexual signal. However, a decline in sexual signalling with age has been recently reported in other traits of vertebrates (e.g. Møller & de Lope 1999; Candolin 2000; Vanpé *et al.* 2007).

In our study, the decrease in the expression of the signal shown by old males might be due to a physiological constraint in the capacity to allocate carotenoids. Testosterone induced an increase of circulating carotenoid levels, supporting recent findings in this (Blas *et al.* 2006; Alonso-Alvarez *et al.* 2008) and other avian species (McGraw *et al.* 2006). However, the effect was only detected in middle-aged males. This apparent constraint could be the result of different proximate mechanisms. First, the absence of a rise in plasma carotenoid levels in old males could have been

due to a lower carotenoid intake. Nevertheless, diet composition and abundance were the same for all birds during the experiment, and body mass change, which may reflect food intake, did not differ between age-classes. Second, old males could have presented lower levels of blood lipoproteins than younger males. Such molecules rise in the presence of high testosterone levels, acting as carriers of carotenoids and limiting their absorption (McGraw *et al.* 2006). Third, the accumulation of oxidative challenges throughout life (Finkel & Holbrook 2000) could have exhausted carotenoid stores in old birds, preventing carotenoid mobilization in blood. On the other hand, in terms of ultimate explanations the suggested constraint may reflect a life-history trade-off between early and late investments, older males exhausting resources involved in signalling during the precedent breeding events.

Oxidative stress is often considered the main mechanism of ageing (Finkel & Holbrook 2000) and immunosenescence (Larbi *et al.* 2007). Here, nonetheless, its role as a potential enhancer of signal reliability was not directly established. Age and testosterone separately influenced lipid peroxidation and plasma antioxidants, respectively (figure 2). The first result indicates damage accumulation during the breeding season in old males, whereas the second one might suggest an exhaustion of antioxidants due to some oxidative challenge promoted by testosterone, such as the one proposed by the oxidation handicap hypothesis (Alonso-Alvarez *et al.* 2007b, 2008). Although we cannot disregard the presence of higher oxidative stress in old T-males in tissues other than blood, the role of oxidative stress in this signalling context was mostly supported by the carotenoid pathway (see positive correlation between antioxidant status and carotenoid level). Nevertheless, it has been suggested that the antioxidant function of carotenoids could be secondary, and some authors underline their immunostimulatory action (Hartley & Kennedy 2004; Costantini & Møller 2008; Pérez-Rodríguez *et al.* 2008).

The fact that the variability in carotenoid levels contributed to explain the differential effect of testosterone on CMI is an interesting result in the ICHH framework. In agreement with our results, Blas *et al.* (2006) showed that 2-year-old testosterone-implanted partridges did not present impaired CMI, CMI also positively correlated to circulating carotenoids. This led them to suggest that the testosterone-mediated increase in circulating carotenoids could be a protective strategy to counteract the immunosuppressive effect of the hormone (also McGraw & Ardia 2007). Our findings support such a hypothesis. However, the results must be taken with caution because they are basically correlational. An experiment manipulating carotenoid availability is now required to definitively demonstrate this mechanism.

In summary, our results emphasize the necessity of analysing the processes and outcomes of sexual selection from a life-history perspective (Kokko 1997; Getty 2002, 2006; Proulx *et al.* 2002). Furthermore, they suggest that the link between testosterone, immune function and carotenoids in the expression of sexual signals (see Peters 2007) may contribute to maintain signal reliability throughout the lifespan of an individual.

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