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Male reproductive senescence: the price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby

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Summary

1. In animals, male reproduction is commonly a function of sexual attractiveness, based on the expression of sexually dimorphic traits that advertise genuinely the male's quality. Male performance may decline with age because physiological functions underlying sexual attractiveness may be affected by senescence.

2. Here we show that a sexual signal (foot colour) declines with age, due probably to the deleterious effects of oxidative damage.

3. We found that in the blue-footed booby *Sula nebouxii* foot colour during courtship was less attractive in senescent than in middle-aged males.

4. In addition, we increased reactive oxygen species experimentally by immunizing males with lipopolysaccharide, a bacterial cell wall component that induces marked oxidative stress in animals. The immune system activation induced greater lipid peroxidation and invoked changes on colour expression (less attractive), particularly in senescent males.

5. These results support the idea that oxidative stress affects reproductive senescence, and suggest that oxidative damage might be a proximal mechanism underlying age-reproductive patterns in long-lived animals.

Key-words: ageing, life-history evolution, ornamental coloration, oxidative stress, sexual selection.

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Introduction

The mechanisms underlying age-specific rates of reproduction in iteroparous organisms remain a major issue to understanding of life-history evolution (Rose & Bradley 1998; Zera & Harshman 2001; Alonso-Alvarez *et al*. 2004a). Senescence, the progressive loss of function accompanied by decreased survival and reproductive performance that individuals experience with advancing age, is believed to be universal in the life history of age-structured animals (Rose 1991; Kirkwood & Austad 2000) and the assumption of evolutionary theories of ageing (Medawar 1952; Williams 1957; Kirkwood 1977). Reproductive senescence in wild populations was thought to be imperceptible, because extrinsic mortality limits the number of individuals that live long enough to exhibit detectable senescence (Promislow 1991; Kirkwood & Austad 2000). Thus, the understanding of reproductive senescence mechanisms comes from laboratory animals (see Kirkwood & Austad 2000). However, recent analyses in wild birds and mammals show evident declines in female productivity with advancing age (e.g. Clutton-Brock 1988; Bérubé, Festa-Bianchet & Jorgenson 1999; Robertson & Rendell 2001; Weladji *et al*. 2002; Broussard *et al*. 2003; Reid *et al*. 2003), although evidence for male reproductive senescence is scarce (but see McElligot *et al*. 2002; Mysterud, Solberg, & Yoccoz 2005).

Male reproductive success is commonly a function of sexual attractiveness, based often on the expression of sexually dimorphic traits that advertise genuinely the male's quality (Andersson 1994). It has been suggested that oxidative damage is the proximal cause of the genuine information revealed to prospective

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females through male secondary traits (von Schantz *et al*. 1999). For instance, sexual signals that depend on antioxidants, such as carotenoids, may reveal current immunological state (Faivre *et al*. 2003; Alonso-Alvarez *et al*. 2004b), because activation of the immune system produces reactive oxygen species that may result in depletion of bodily antioxidants to balance oxidative stress at the expense of the expression of sexual traits (Blount *et al*. 2003; Faivre *et al*. 2003; Alonso-Alvarez *et al*. 2004b; Grether *et al*. 2004). Nevertheless, because older animals accumulate and are less able to prevent oxidative damage, due probably to a decline of antioxidant defences and/or repair mechanisms (Beckman & Ames 1998; Tian, Cai & Wei 1998; Finkel & Holbrook 2000), older individuals appear more susceptible to oxidative injury produced by immune activation. Thus, particularly in long-lived animals, age reproductive patterns may be related to senescence of oxidative-dependent physiological functions underlying sexual signal expression. Furthermore, individuals may adopt different mating strategies as they age (Yoccoz *et al*. 2002), modifying their relative antioxidant investment to different components of reproductive effort, including sexual attractiveness (Alonso-Alvarez *et al*. 2006).

Surprisingly, however, despite the fact that the role played by oxidative stress in the age-related loss of function has been recognized for a long-time (Harman 1956), the link between male reproductive senescence and age-associated decline in oxidative-dependent sexual signals has not been investigated previously. One possible reason is that it is believed that females may prefer males with ornaments that signal older age if survival relates to genetic quality (Trivers 1972; Manning 1985), and in fact males of several species increase their sexual ornamentation as they grow older (Brooks & Kemp 2001). Nevertheless, the assumption that age indicates fitness is frequently not achieved (Hansen & Price 1995). In many long-lived animals, elderly males are often least fertile (Kidd, Eskenazi & Wyrobek 2001), accumulate higher deleterious germline mutations (Hansen & Price 1999; Radwan 2003) and could have lower genetic quality when antagonistic pleiotropic effects are present (Hansen & Price 1995; Brooks & Kemp 2001). In addition, in species with bi-parental care, senescent males are probably poorer food-providers than younger males if condition declines with age (Wedell & Ritchie 2004). In these circumstances, old age should be penalized during female mate choice among males, as it may reduce offspring number and quality (Jones, Balmford & Quinell 2000; Saino, Ambrosini, & Møller 2002; see Radwan 2003 for a review).

In this study, we first used the blue-footed booby *Sula nebouxii* (Milne-Edwards) to test the prediction that sexual signals decline with age, and then to investigate whether immune activation leads to a rise in oxidative damage with consequences on sexual attractiveness on different age groups. Blue-footed boobies are long-lived birds with reproductive senescence; a long-term study indicates that the number of fledglings varies with male age and declines steeply after the tenth year of life (Velando, Drummond & Torres 2006a). Foot colour, a sexually selected trait, varies from light green to dull blue, and females prefer light green feet (Torres & Velando 2003). The expression of this dynamic trait is modulated by carotenoids (diet-derived pigments with antioxidant and immunomodulatory properties; Blount *et al*. 2003; McGraw & Ardia 2004), and reflects genuinely some aspect of the underlying condition of the immune system (Velando, Beamonte-Barrientos & Torres 2006b). Thus, male foot colour correlates positively with *in vivo* cell-mediated immune response, and supplementation of dietary carotenoids invokes increases in cell-mediated immune function and foot colour (Velando *et al*. 2006b), suggesting that oxidative stress is a limiting factor for both foot colour and immune function. Interestingly, colourful integuments continuously demand antioxidant pigments (Alonso-Alvarez *et al*. 2004b), and individuals should optimize pigment allocation according to their condition to balance the trade-off between physiological costs and mating benefits. For blue-footed booby males, continuous allocation of antioxidant pigments to foot colour during courtship may affect the probability of being cuckolded (Torres & Velando 2003) and achieving extra-pair paternity (Osorio-Beristain & Drummond 1998); furthermore, male foot colour influences female investment to eggs (Velando *et al*. 2006b). Thus, males should mobilize high flux of pigments into foot colour as laying approach. As older individuals are possibly more susceptible to oxidative injury (Tian *et al*. 1998; Finkel & Holbrook 2000), the optimal strategy of pigment deposition into integuments of older and younger blue-footed booby males should differ.

To assess whether sexual attractiveness is affected by immunologically induced oxidative damage, we injected lipopolysaccharide (LPS) in courting males and evaluated the effects by comparing changes in plasma levels of immunoglobulins (IgG) and malondialdehyde (a marker of lipid peroxidation; Armstrong 1998), and changes in foot colour. LPS mimics a bacterial infection without the negative effects of pathogens (Bonneaud *et al*. 2003), and is known to increase the formation of reactive oxygen species and lipid peroxidation products, such as superoxide anions and peroxides, inducing remarkable oxidative stress in animals (e.g. Wiesel *et al*. 2000; Escames *et al*. 2003). The high flux of oxidants produced by the immune activation should be counteracted by the mobilization of antioxidant scavengers at the expense of the expression of sexual traits (Alonso-Alvarez *et al*. 2004b). We predict that immune activation leads to a rise in oxidative stress, with major consequences on the pigment availability to sexual traits for elderly males.

Methods

The study was carried out in the breeding colony of the blue-footed booby *Sula nebouxii* at Isla Isabel, Nayarit,

Mexico from January to February 2004. All the birds included in this study were banded in our long-term study of the blue-footed booby (Drummond, Torres & Krishnan 2003). In every season since 1988, we registered all breeding attempts and marked individually all fledglings and adults breeding in the study area (Drummond *et al*. 2003).

EXPERIMENTAL IMMUNE ACTIVATION WITH LPS

During courtship, 50 males were captured and assigned randomly to one of two treatment groups. The experimental group had their immune system activated by intraperitoneal injection of 0·1 mg of LPS (LPS of *Escherichia coli*, serotype 055:B5) in 1 mL of phosphate-buffered saline solution (PBS). To avoid provoking breeding desertion (Bonneaud *et al*. 2003), we chose a concentration (0·08 mg/kg of body weight) lower than concentrations used to activate the immune system of poultry (0·5–5 mg/kg of body weight; Nakamura *et al*. 1998; Xie *et al*. 2000).The control group was injected with an identical volume of PBS. Prior to the experimental manipulation, we measured the foot colour and sampled blood (1 mL) from the brachial vein to assess plasma levels of IgG and lipid peroxidation. All blood samples were kept on ice until centrifugation (less than 1 h later). The plasma was separated and preserved immediately on liquid nitrogen until laboratory analysis. Seven days after manipulation birds were recaptured (16 controls and 21 experimentals) and we measured foot colour and obtained a second blood sample. On average, males were handled for less than 8 min to minimize stress during capture.

Foot colour was measured by taking three measures on the foot web using a spectrophotometer (Minolta CM-2600d). Reflectance spectrum was determined from 360 to 740 nm wavelength at 10-nm intervals. Because the highest visual sensitivity in blue-footed boobies occurs between 460 and 620 nm (Reed 1987), we analysed the chroma of foot colour in this range (hereafter green chroma) estimated as the sum of the reflectance between 460 and 620 nm divided by the sum of the reflectance between 360 and 740 nm (total spectrum reflectance).

Based on the results of the longitudinal analysis, which show that around 10 years of age within individual males reproduction success decreases (Velando *et al*. 2006a), we classified experimental males into two age classes: 3–9 years (middle-aged males, $n = 35$) or ≥ 10 years (senescent males, $n = 15$). Although all males in the experiment had been ringed as part of the long-term study (most of them as fledglings), 36% of them were ringed at recruitment, which occurs typically at age 3–7 years (Drummond *et al*. 2003). Males banded at recruitment could not be aged precisely, but each one could be assigned to one of our two age classes: the eight males that recruited between 1993 and 1997 were considered senescent, and the 10 males that recruited between 2002 and 2004 were considered middle-aged. Males of control and experimental age groups included in our experiment did not differ in the number of years they have mated previously with the same partner (mean = 0·27, range 0–2 previous years with the same partner; $P > 0{\cdot}48$ in all cases). Moreover, only five males (two senescent and three middle-aged) maintained the pair bond with the same female from the previous reproductive season (2003).

ASSAYS FOR LIPID PEROXIDATION AND IGG CONCENTRATION IN PLASMA

Oxidative damage was estimated as lipid peroxidation concentration using 100 μL of plasma by thiobarbituric acid reactive substances (TBARS) technique (Oxi-Tek TBARS assay kit). Lipid peroxidation, a major indicator of oxidative damage, was detected by the formation of reactive malondialdehyde products, measured by spectophotometry (Armstrong 1998). TBARS were expressed as malondialdehyde equivalents estimated from a standard curve.

We assessed plasma IgG using an enzyme-linked immunosorbent assay (Janeway & Travers 1999). Briefly, microtitre plates were coated with antigen [antichicken IgY (IgG)] by overnight incubation and blocked with PBS/bovine serum albumin (BSA). Duplicated samples (50 μL each) from males were diluted 1 : 128 000 with PBS/BSA, added and incubated at 37 °C. Peroxidase conjugate secondary antibodies (antichicken IgG diluted 1 : 2000) were added to the wells and incubated at 37 °C; the enzyme-substrate reaction was achieved by citrate buffer and the optical density was determined using a microplate reader equipped with 450 nm filters. For each plate, a standard curve was obtained using dilutions of purified immunoglobulin chicken IgG. Amounts of IgG are expressed in equivalent mg/mL of chicken IgG.

STATISTICAL ANALYSES

Changes in IgG levels, lipid peroxidation and colour (expressed as the difference before and 7 days after an intraperitoneal injection of LPS or PBS) were compared by mixed linear models using PROC MIXED in SAS (Littell *et al*. 1996), with individual birds as subject factor, the age and immune treatment as factors and the repeated measures at different times (initial and final) as within-subject factor.

Differences in sample sizes reflect missing values due to, for instance, reduced blood volume. All the statistical analyses were performed using SAS software (SAS Institute 2001). Data are expressed as mean ± SE.

Results

Initial levels of IgG, lipid peroxidation markers and foot colour (green chroma) did not differ between treatments ($P > 0{\cdot}12$ in all cases). Prior to the manipulation, senescent males showed duller foot colour

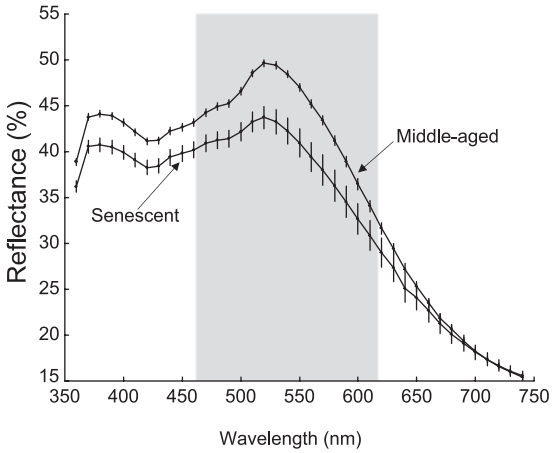

Fig. 1. Reflectance curves of blue-footed booby foot colour of middle-aged (3–9 years, $n = 35$) and senescent males (≥ 10 years, $n = 15$) during courtship. As blue-footed boobies have a maximum visual sensitivity at 460–620 nm (shadow area), the chroma in this wavelength range (green chroma) of foot colour was analysed. Data are expressed as means ± SE.

(0·508 ± 0·004) than middle-aged males (0·521 ± 0·001; $F_{1,48} = 15{\cdot}45$, $P < 0{\cdot}001$; Fig. 1), but concentrations of IgG and lipid peroxidation products were initially similar for the age classes (chicken IgG equivalents in mg/mL, senescent males 0·42 ± 0·04, middle-ages 0·36 ± 0·02; $F_{1,45} = 1{\cdot}61$, $P = 0{\cdot}21$; lipid peroxidation expressed as malondialdehyde equivalents in nmol/mL, senescent males 8·27 ± 1·05, middle-ages 8·48 ± 1·13; $F_{1,41} = 1{\cdot}79$, $P = 0{\cdot}13$).

Seven days after immune treatment, IgG levels increased significantly (0·14 ± 0·03 mg/mL chicken IgG equivalents) in males injected with LPS (mixed linear model, $F_{1,32} = 8{\cdot}95$, $P = 0{\cdot}005$; Fig. 2a), but no differences between age groups were found (age group, $F_{1,32} = 1{\cdot}06$, $P = 0{\cdot}31$; treatment × age, $F_{1,32} = 0{\cdot}01$, $P = 0{\cdot}92$; Fig. 2a), suggesting that both age groups mounted a similar response. The activation of the immune system induced a strong rise in markers of lipid peroxidation (mixed linear model; $F_{1,32} = 6{\cdot}53$, $P = 0{\cdot}02$), particularly in senescent males, although the interaction fell short of statistical significance ($F_{1,32} = 2{\cdot}72$, $P = 0{\cdot}08$; Fig. 2b). Thus, lipid peroxidation of control males (senescent and middle-aged) decreased 5·27 ± 1·32 nmol/mL (malondialdehyde equivalents) while lipid peroxidation of LPS males increased 1·28 ± 2·25 nmol/mL. When only males in the LPS group were considered, changes in lipid peroxidation and IgG were independent in middle-aged males ($\beta = -0{\cdot}02$); in contrast, in senescent males there was a rise of lipid peroxidation as IgG increased [$\beta = 0{\cdot}60$, Fig. 2c, analysis of covariance (ANCOVA), age × IgG change, $F_{1,14} = 9{\cdot}74$, $P = 0{\cdot}008$; initial lipid peroxidation, $F_{1,14} = 19{\cdot}42$, $P = 0{\cdot}001$]. Hence, with a similar IgG increase, senescent males suffered a greater rise in lipid peroxidation products than middle-aged males.

Overall, LPS males displayed a significant change in foot colour (mixed linear model; $F_{1,32} = 5{\cdot}23$, $P = 0{\cdot}03$),

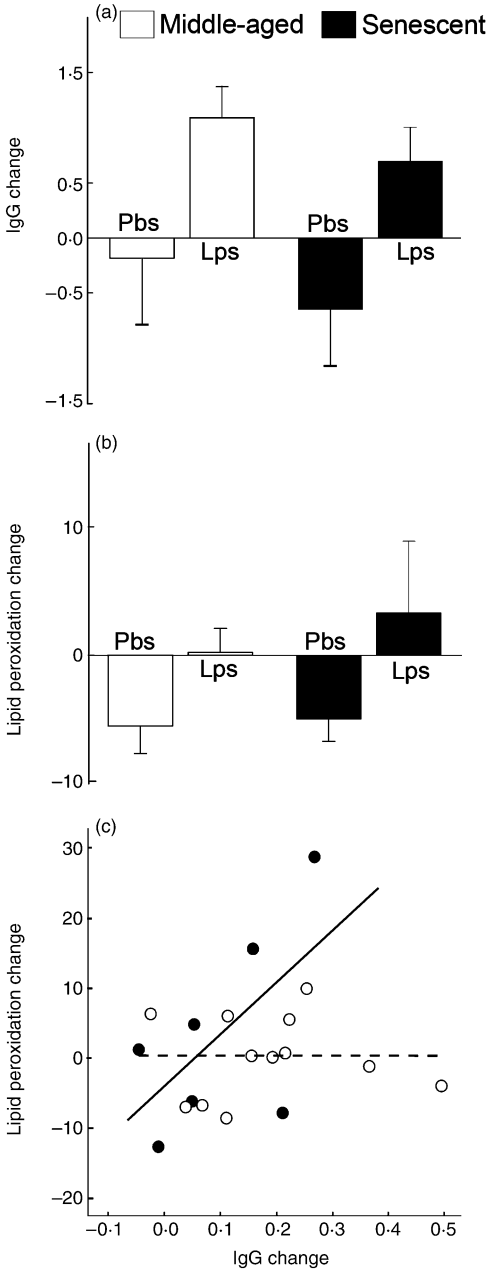

Fig. 2. Effects of immune activation on middle-aged (open symbols) and senescent males (closed symbols); changes are expressed as the difference before and seven days after an intraperitoneal injection of LPS or PBS. (a) Change in IgG concentration (chicken IgG equivalents in mg/mL; middle-aged, $n = 22$; senescent males $n = 14$) (b) change in oxidative damage (malondialdehyde equivalents in nmol/mL; middle-aged, $n = 20$; senescent males $n = 14$); (c) relationship between changes in oxidative damage (malondialdehyde equivalents in nmol/mL) and IgG changes (chicken IgG equivalents in mg/mL) in the LPS group (solid line: senescent males, dashed line: middle-aged males).

and the interaction between the treatment and the age group was significant ($F_{1,32} = 4{\cdot}04$, $P = 0{\cdot}027$; Fig. 3); immune activation had no effect on middle-aged males (Tukey test, $P = 0{\cdot}28$), but a strong effect on senescent males ($P = 0{\cdot}0026$). Thus, control senescent males increased foot colour (Fig. 3) parallel to the

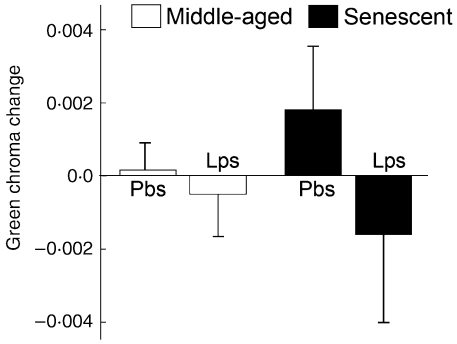

Fig. 3. Effects of immune activation on foot colour changes expressed as the difference before and 7 days after an intraperitoneal injection of LPS or PBS on middle-aged (open bars; $n = 23$) and senescent males (closed bars; $n = 14$). Foot colour change is expressed as the chroma change in the green range (sum of the reflectance between 460 and 620 nm divided by the total spectrum) corrected by initial values.

decrease in lipid peroxidation products experienced by this group (Fig. 2b), while senescent males that were challenged immunologically suffered a decrease in foot colour (Fig. 3). Furthermore, in senescent males the relationship between foot colour and changes in lipid peroxidation was affected by the immune challenge (ANCOVA; treatment × lipid peroxidation change, $F_{1,10} = 6{\cdot}19$, $P = 0{\cdot}032$). In LPS senescent males, the immune challenge provoked a parallel increase in lipid peroxidation and a decrease in foot colour ($\beta = -0{\cdot}39$). In contrast, in control senescent males, foot colour increased when the change in lipid peroxidation was close to zero ($\beta = 0{\cdot}28$). Additionally, senescent males which had high levels of oxidative damage initially showed a decrease in this variable at the end of the 7 days ($r = -0{\cdot}93$, $P = 0{\cdot}002$).

Discussion

In the blue-footed booby, foot colour decreases with age indicating senescence in a trait under sexual selection. Overall, the results suggest that ageing incurs deterioration in the expression of a male sexual signal, whereby older males are incapable of being as attractive as younger males. In this species, a decrease in reproductive success with ageing should be expected due to reduced sexual attractiveness. Accordingly, longitudinal data from two cohorts from our long-term study indicate that, after initial increase, reproductive success of male boobies declines progressively with age (Velando *et al*. 2006a).

Theory predicts that females should discriminate against older males when mating with older males reduces offspring number or quality (e.g. Jones *et al*. 2000; Saino *et al*. 2002). In the blue-footed booby females prefer mates with brighter foot colour, a signal of good nutritional condition (Torres & Velando 2003; Velando *et al*. 2006b). Thus, by discriminating against males with dull foot colour, females may avoid older males. A cross-fostering experiment showed that chick growth correlates with foot colour of the foster father and, to some extent, with the foot colour of the genetic father, suggesting that foot colour may indicate parental contribution and some genetic quality (Velando, Torres & Espinosa 2005). The dull foot colour of senescent blue-footed booby males may possibly be an indicator of their reduced ability to provide parental care (Wedell & Ritchie 2004), their low fertility (Kidd *et al*. 2001) and/or their lower genetic quality (Hansen & Price 1999; Radwan 2003).

The results from the experiment suggest that senescent males apparently suffered a cost of mounting an immune response in terms of oxidative damage and sexual attractiveness. At the onset of our experiment, senescent and middle-aged males had similar levels of IgG and lipid peroxidation products, and after an immune challenge both age groups mounted a similar response (change in IgG). The increase in lipid peroxidation products by LPS challenged senescent males was greater than in middle-aged males, but the difference was not significant ($P = 0{\cdot}08$). Moreover, immune challenge provoked a rise in plasma IgG and, although weak, there was a positive relationship between changes in lipid peroxidation and IgG in senescent, but not in middle-aged males. The magnitude of the IgG increase may reflect the effect of the immune challenge and/or indirect effects if LPS increased the probability of infection risk; in any case, IgG increase probably indicates the immune activity (directly or indirectly related to LPS injection). Thus, the results suggest that middle-aged, but not senescent males, were able to defend against radical attack produced by the immune response. In laboratory mice, lipid peroxidation products increase strongly after the administration of LPS, but the production of reactive oxygen species is higher in older animals (Escames *et al*. 2003), which may explain why aged mice are typically more sensitive to the negative effects of LPS (Tateda *et al*. 1996). Although the mechanisms underlying the ageing process are poorly understood, our results suggest that blue-footed booby senescent males incur higher costs by mounting an immune response.

Activation of the immune system resulted in a decrease in the expression of male foot colour, a carotenoid-dependent sexual signal (Velando *et al*. 2006b). These results are consistent with experiments showing a trade-off in antioxidants (carotenoids) allocation between a sexual signal and the immune system (Faivre *et al*. 2003; Alonso-Alvarez *et al*. 2004b). In our study, changes in lipid peroxidation indicate clearly that the immune activation directly increased oxidative damage, and in turn this provoked a parallel decrease in the sexual signal, particularly in senescent males. Overall, the results suggest that oxidative stress is one of the proximal mechanisms of the trade-off between sexual signals and immune competence (von Schantz *et al*. 1999).

In our study, oxidative stress probably decreased in control situation between initial and final captures as

indicated by the reduction of lipid peroxidation products in plasma. Middle-aged males showed small variation in lipid peroxidation and foot colour. In contrast, senescent males suffering a rise of immune-induced oxidative damage (LPS group) suffered a decrease in foot colour compared to control senescent males. Overall, these results suggest that middle-aged and senescent males differ in their pigment allocation to foot colour, due probably to a decline with age in their ability to deal with oxidative stress. Middle-aged males can apparently afford the maintenance (with slight variations) of bright blue feet after an immune challenge. Senescent males under conditions of higher oxidative stress (as occurs in LPS group) probably allocate relatively more antioxidant resources to somatic defence, while those under conditions of lower oxidative stress (as occurs in the control group) increase pigment deposition to foot colour.

In the blue-footed booby, the maintenance of colourful feet throughout the courtship period, as did middle-aged males (control and LPS), should benefit males by increasing paternity probability (extra and intra) and female investment (Torres & Velando 2003; Velando *et al*. 2006b). Interestingly, senescent males in the control group displayed dull colour, probably losing some benefits, but increased foot colour as laying approached. In the blue-footed booby sexual attractiveness prior to laying is especially important to reduce the probability of being cuckolded (Osorio-Beristain & Drummond 1998; Torres & Velando 2003) and to increase maternal investment into eggs (Velando *et al*. 2006b). In this line of argument, females paired with control senescent males laid greater eggs (total egg volume of two egg clutches $116{\cdot}3 \pm 1{\cdot}0$ cm^3, $n = 6$) than did females paired with LPS senescent males ($113{\cdot}2 \pm 1{\cdot}4$ cm^3, $n = 5$), although the differences were not significant ($P = 0{\cdot}09$). In addition, our results suggest that senescent males optimize pigment mobilization decisions by increasing foot colour as laying approached. However, despite the increase in foot colour, the absolute values of green chroma of control senescent males ($0{\cdot}511 \pm 0{\cdot}004$) were still inferior to those of middle-aged males ($0{\cdot}519 \pm 0{\cdot}001$), suggesting a generally lower performance at facing the oxidative costs of self-maintenance.

Evolutionary theory explains senescence as a consequence of the force of natural selection declining with age (Medawar 1952; Williams 1957). In theory, female preference based on age can actually influence the evolution of ageing (Beck *et al*. 2002; Promislow 2003). By choosing to mate preferentially with older males, females can increase the frequency of genes for high viability and the strength of selection acting at late ages, thus increasing the life span over evolutionary time (Beck *et al*. 2002; Promislow 2003). In contrast, female preferences for middle-aged males may accelerate senescence because selection should favour highest investment in the age classes more likely to contribute to male reproduction (Beck *et al*. 2002). We suggest that senescence in sexual traits modulated by oxidative stress (von Schantz *et al*. 1999), such as pigment-demanding integuments, may be common, playing a key role in the evolution of male age-reproductive patterns in long-lived animals and, thereby, influencing their social structure and population dynamics in nature (Coulson *et al*. 2001).

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