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SHORT COMMUNICATION

Senescent males carry premutagenic lesions in sperm

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Abstract

As organisms age, DNA of somatic cells deteriorates, but it is believed that germ cells are protected from DNA-damaging agents. In recent years, this vision has been challenged by studies on humans indicating that genomic instability in germ cells increases with age. However, nothing is known about germ line senescence in wild animals. Here, we examine DNA damage in sperm of a wild vertebrate, the blue-footed booby *Sula nebouxii*. One of the major types of premutagenic DNA damage generated by oxidative stress (a proximal cause of ageing) is loss of single bases resulting in apurinic/apyrimidinic sites (AP sites). We examined AP sites in the sperm of known-age males sampled during courtship on Isla Isabel, Mexico. We show that damage to the DNA of sperm increases with age of male blue-footed boobies. Moreover, we found that sexual attractiveness (foot colour) declines with age and is correlated with germ line damage of senescent males. By choosing attractive males, females might reduce the probability of their progeny bearing damaged DNA. This study reports the first evidence of senescence in the germ line of a wild vertebrate and future studies should investigate whether this burden of senescence is sidestepped by potential sexual partners.

Introduction

DNA molecules carry the genetic information of an organism. Because maintaining the integrity of the genome is of vital importance, a range of mechanisms has evolved to overcome the mutagenic and lethal effects of damage to DNA (Vijg, 2007). In multicellular organisms, an early differentiation event segregates immortal germ cells from mortal soma (Weismann, 1881; Kirkwood, 1977). As organisms age, the DNA of somatic cells accumulates errors, but the germ line must be maintained to preserve offspring viability. Somatic deterioration in ageing animals may arise from allocation of resources to reproduction at the expense of repair and maintenance (Kirkwood & Austad, 2000). In contrast, it is believed that germ cells are adequately protected from DNA-damaging agents and constantly rejuvenated (Vijg, 2007).

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In recent years, this vision has been challenged by studies on humans indicating that genomic instability in germ cells increases with age (e.g. Wyrobek *et al.*, 2006; Schmid *et al.*, 2007; Sartorius & Nieschlag, 2010). Some evidence in animals suggests that the sperm from old males deteriorates, sperm from aged males have lower performance (velocity and motility: Møller *et al.*, 2009; Dean *et al.*, 2010), mating with old males may reduce female fecundity (Jones & Elgar, 2004; Jones *et al.*, 2007; Hale *et al.*, 2008) and offspring fathered by aged males may have developmental defects (Price & Hansen, 1998; Serre & Robaire, 1998). However, nothing is known about senescence in germ line DNA of wild animals.

The DNA in germ cells is destined for the next generation and the ova have numerous DNA repair enzymes that prevent the build-up of dangerous mutations (Vogel *et al.*, 1985; Agrawal & Wang, 2008). In contrast, sperm DNA is prone to damage because of the higher number of divisions that germ cells undergo, resulting in a higher mutation rate in spermatogenesis than in oogenesis (Ellegren, 2007). DNA damage in sperm persists because there is little, if any, post-meiotic

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repair in males (Vogel *et al.*, 1985). Spermatozoa are particularly vulnerable to oxidative injury, which probably increases as animals age (Velando *et al.*, 2008). One of the major types of DNA damage generated by oxidative stress is loss of single bases, resulting in apurinic/apyrimidinic sites (AP sites; Loeb & Preston, 1986). Oxidation of DNA in the male germ line may be a prominent force in the evolution of ageing, and also in mate choice and sexual signalling (Pizzari *et al.*, 2008; Velando *et al.*, 2008).

Here, we examined DNA damage in sperm of a wild vertebrate, the blue-footed booby *Sula nebouxii*. Males of this species display age-related somatic deterioration resulting in declining physiological functions, sexual attractiveness (foot colour) and, consequently, reproductive success after age 10 years (Velando *et al.*, 2006a, 2010; Torres & Velando, 2007). We predicted an increase in germ line DNA damage with age, if sperm is inadequately protected from damaging agents as males age. Because females could benefit by assessing premutagenic damage levels of potential mates and might do this by responding to foot colour (a signal that affects females' responsiveness to courting males, Torres & Velando, 2003; Velando *et al.*, 2006b), we tested whether male foot colour is correlated with DNA damage level in sperm (Velando *et al.*, 2008; see also Blount *et al.*, 2001).

Materials and methods

The study was carried out in the blue-footed booby breeding colony on Isla Isabel, Nayarit, Mexico, in 2005. All birds included in this study were previously banded in a long-term study: every season since 1988, all breeding attempts in the study area were registered and all fledglings were individually marked (Drummond *et al.*, 2003). During the courtship period in 2005, 42 males were captured by hand at night. Captured birds were sampled for sperm, weighed and their foot colour was measured using a spectrophotometer (MINOLTA CM-2600d). Sperm was obtained by cloacal massage and transferred to a cryovial, which was kept on ice during < 1 h then preserved on liquid nitrogen until laboratory analysis.

We analysed chroma of foot colour as the proportion of reflectance between 460 and 620 nm (range of highest visual sensitivity; hereafter, green chroma; Velando *et al.*, 2006a). In this species, males displaying greener feet during courtship are more attractive to females (Torres & Velando, 2003; Velando *et al.*, 2006a). Based on the results of longitudinal analyses showing that male reproductive success decreases at around age 10 years (Velando *et al.*, 2006b), we classified sampled males as middle-aged (3–10 years) or senescent (> 10 years). In the study colony, few males live to age 17 years (Kim *et al.*, 2007). Although all focal males had been ringed as part of the long-term study (most of them as fledglings), 14% of them were ringed at recruitment, which typically occurs at age 3–7 years (95% of 1180 ringed recruits in the data base). These males were assigned to one of two age classes: three that recruited between 1993 and 1994 were considered old and three that recruited between 2002 and 2004 were considered middle-aged. Laying dates of all 35 focal males that eventually incubated a clutch were recorded by routine monitoring of the study area until the end of reproduction (Drummond *et al.*, 2003).

Sperm DNA was extracted by a Chaotropic NaI-based method, as recommended by European Standards Committee on Oxidative DNA Damage (ESCODD), which minimizes DNA artifactual oxidation during extraction (Gedik *et al.*, 2005). The amount of isolated DNA was determined by high-sensitivity fluorescent assay (Quant-iT™ High-Sensitivity DNA Assay kit; Invitrogen, Barcelona, Spain). In 34 of 42 samples, we isolated enough DNA to estimate DNA damage. Protein contamination was checked using the absorbance ratio A260/A280; an absorbance ratio over 1.6 was acceptable (> 1.85 in all samples). DNA damage was estimated as apurinic-apyrimidinic (AP) sites using a biotin-labelled reagent specific for the aldehyde group in the ring-open form of AP site, designated as the aldehyde reactive probe (ARP; Oxidative DNA Damage Quantitation kit-AP sites; Cell Biolabs, San Diego, CA, USA). ARP specifically binds to AP sites in isolated genomic DNA, and the biotin molecular in ARP can then be detected calorimetrically using a streptavidin-enzyme conjugate. In brief, a known concentration of the purified DNA isolated from sperm was dissolved at 100 μg mL^{-1} in Tris and EDTA (TE) buffer and incubated with the ARP solution at 37 °C for 1 h. The ARP-labelled DNA was then ethanol precipitated, and the DNA pellet was suspended in TE buffer. ARP-labelled DNA was fixed in a plate and incubated with streptavidin-enzyme conjugate, which results in a yellowish complex that can be measured spectrophotometrically at 450 nm (Microplate reader 550; Bio-Rad, Richmond, VA, USA). The quantities of AP sites in DNA samples were determined by comparing their absorbance with a standard curve (0–8 AP per 10^4 pb) generated by increasing or decreasing the amount of DNA standard containing predetermined AP sites (four AP sites per 10^4 pb).

The number of AP sites in the sperm from 34 males was analysed with a general linear model. The initial model included age, body mass, previous breeding effort (estimated as the number of previous breeding attempts) and breeding status (whether the bird established a clutch after capture, during the same year). All main factors and two-way interactions were included in the initial model, and then nonsignificant interactions and main terms were dropped sequentially to simplify the model (α level was set at 5%). We also ran the same model including laying date or the elapsed time between capture and laying date in the subset of males that established a clutch (n = 31). Additionally, we explored whether DNA damage was correlated with foot colour by including this variable in the model.

Results

DNA damage in the sperm of courting males increased with increase in previous breeding effort ($r = 0.35$ $P = 0.044$). In the final model only breeding status and age class were significantly related to DNA damage (Table 1; breeding effort was not significantly related to DNA damage after controlling for age; $P > 0.6$). Senescent males (> 10 years) had 85% more AP sites than middle-aged males (Fig. 1; Table 1). The three males captured during courtship that did not establish a clutch showed significantly lower DNA damage in sperm than breeders (Table 1). Neither body mass, laying date nor time from capture to laying (in the analyses on the subset of males that established a clutch) was related to the proportion of AP sites in sperm DNA ($P > 0.35$).

Senescent males displayed duller foot colour (green chroma) than middle-aged males (green chroma: 0.512 ± 0.002 vs. 0.524 ± 0.001; $t_{41} = 5.03$, $P < 0.001$). Interestingly, foot colour and age interacted significantly in their relationship to DNA damage in sperm (Fig. 2; $F_{1,30} = 8.86$, $P = 0.006$). The number of AP sites declined with foot colour in senescent males ($r = -0.82$, $P = 0.002$) but not in middle-aged males ($r = 0.16$, $P = 0.46$). Similar results were obtained when the analysis was restricted to breeding males (age*foot colour, $F_{1,27} = 5.91$, $P = 0.022$).

Table 1 Summary of the final general linear model on DNA damages in sperm of blue-footed boobies.

Source	Estimate (SE)	*F*	df	*P*
Intercept	6.16 (0.88)			
Breeding status (nonbreeder)	−2.16 (1.03)	5.55	1	0.025
Age class (middle-aged)	−4.02 (1.71)	4.36	1	0.045
Error			31	

Discussion

Our study indicates that damage in the DNA of sperm, measured as AP sites, increases as a male blue-footed booby ages. Because AP sites are DNA lesions that inhibit DNA replication and promote base substitutions, mutations and loss of genetic integrity (Loeb & Preston, 1986), progeny of senescent males is at high risk of genetic disorders (Sartorius & Nieschlag, 2010). Alternatively, our results could be explained by post-meiotic sperm senescence (Pizzari *et al.*, 2008) if senescent males copulate less frequently than middle-aged males. However, copulation rates of this booby are not related to male age (R. Beamonte-Barrientos, H. Drummond, A. Velando & R. Torres, unpublished data). As far as we know, this is the first evidence of senescence in the germ line of a wild vertebrate and it challenges the idea that the germ line DNA of wild animals is effectively protected against DNA-damaging agents during senescence.

The loss of single bases, resulting in AP sites, is a prominent insult to cellular DNA that can block the progress of the DNA replication apparatus and cause mutations (Gentil *et al.*, 1984). Reduced fertility of old males (Jones & Elgar, 2004; Jones *et al.*, 2007; Hale *et al.*, 2008) may prevent fertilization by damaged sperm. Nevertheless, even with significant levels of DNA damage, sperm retains the ability to fertilize oocytes, although subsequent development may be altered (e.g. White *et al.*, 2008; Ruiz-López *et al.*, 2010). Interestingly, in the blue-footed booby, offspring resemblance in a life history trait is depressed in offspring fathered by old males (Kim *et al.*, 2010), suggesting that senescent decline is coupled to an age-related change on the

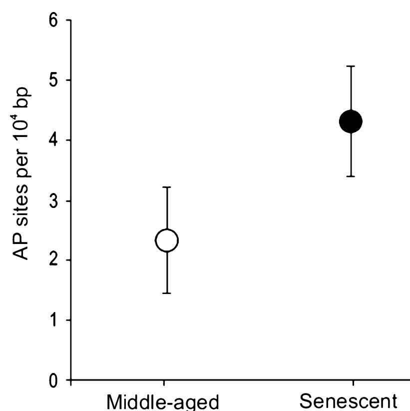

Fig. 1 DNA damages in sperm of blue-footed boobies, measured as the number of apurinic/apyrimidinic sites, in relation to age classes: middle-aged (3–10 years; $n = 23$) and senescent males (> 10 years; $n = 11$); (mean ± SE).

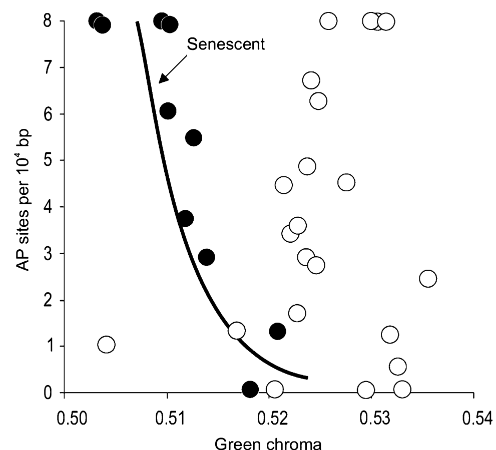

Fig. 2 Number of apurinic/apyrimidinic sites in relation to sexual attractiveness (green chroma of the foot) of middle-aged (open symbols) and senescent males (closed symbols). Line shows an adjusted exponential curve on senescent males.

genetic level. Thus, overall these results point to a probable accumulation of de novo mutations in the germ line cells of the offspring of senescent males (see also Ortego *et al.*, 2008).

Older male boobies, with accumulated reproductive experience, have more DNA lesions in the germ line than middle-aged males. Similarly, we previously found that somatic deterioration, indicated as a decline in sexual attractiveness, increases with age and is related to previous breeding effort (Velando *et al.*, 2010). Thus, both somatic and germ line deterioration might be explained by reproductive investment. Nevertheless, in the present study, effects of previous reproductive effort could not be separated from intrinsic age effects. Note that our results could also be explained by males with higher damage in sperm DNA possibly living longer. Lower sperm DNA damage in nonbreeders might suggest that enhanced courtship or copulation rates increase damage, but this result should be taken with caution because reduced sample size in the nonbreeders group. Effects of age and reproductive effort on damage to sperm DNA should be explored with longitudinal studies.

Recently, sexual ornaments, particularly carotenoid-based colourful traits, have been found to signal sperm protection against oxidative stress (Helfenstein *et al.*, 2010; Pike *et al.*, 2010). Sexual attractiveness of the blue-footed booby (carotenoid-dependent foot colour) declines with age (Torres & Velando, 2007; Velando *et al.*, 2010) and, interestingly, is correlated with germ line damage in senescent males. This result may also arise from colourful males with germ line DNA damage having shorter life expectancy, and this possibility should also be explored by longitudinal analyses. In any case, females could circumvent saddling their progeny with damaged DNA by avoiding unattractive old males, as these are more likely to carry age-related damage. On average, middle-age males had less germ line damage and displayed more colourful feet than senescent males but their foot colour was not correlated with their germ line damage. Importantly, these results suggest that sexual ornamentation may be more honest in ageing animals, as theory predicts (Proulx *et al.*, 2002).

In conclusion, we found evidence of senescence in the germ line of a wild vertebrate, suggesting a possible role of senescent males on population mutation load (i.e. the continuous presence of deleterious mutations that causes a reduction in mean fitness). Furthermore, premutagenic damage in germ line DNA was related to the foot colour of senescent males. Because DNA integrity in germ cells is important for preserving the lineage of the reproductive cell, selection should favour mechanisms that purge, repair or avoid damaged germ cells. The role of mate choice in selecting against propagation of deleterious mutations from ageing animals needs to be explored in wild animals (Radwan, 2004; Velando *et al.*, 2008; Cotton, 2009; Whitlock & Agrawal, 2009).

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