

Historical mutation rates predict susceptibility to radiation in Chernobyl birds

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Abstract

Extreme environmental perturbations are rare, but may have important evolutionary consequences. Responses to current perturbations may provide important information about the ability of living organisms to cope with similar conditions in the evolutionary past. Radioactive contamination from Chernobyl constitutes one such extreme perturbation, with significant but highly variable impact on local population density and mutation rates of different species of animals and plants. We explicitly tested the hypothesis that species with strong impacts of radiation on abundance were those with high rates of historical mutation accumulation as reflected by cytochrome *b* mitochondrial DNA base-pair substitution rates during past environmental perturbations. Using a dataset of 32 species of birds, we show higher historical mitochondrial substitution rates in species with the strongest negative impact of local levels of radiation on local population density. These effects were robust to different estimates of impact of radiation on abundance, weighting of estimates of abundance by sample size, statistical control for similarity in the response among species because of common phylogenetic descent, and effects of population size and longevity. Therefore, species that respond strongly to the impact of radiation from Chernobyl are also the species that in the past have been most susceptible to factors that have caused high substitution rates in mitochondrial DNA.

Introduction

Evolution on long time scales depends on the ability of organisms to cope with extreme environmental perturbations (Hoffmann & Parsons, 1997). For example, mass extinctions have occurred several times, leaving only a small, but nonrandom sample of species to survive (Lawton & May, 1995). Mass extinctions during the Permian (e.g. Bowring *et al.*, 1998; Raup & Sepkoski, 1982) and the boundary between the Cretaceous and the Tertiary (e.g. Alvarez *et al.*, 1980; Jablonski, 1986) constitute such events. The factors that contribute to

such nonrandomness are of major importance for understanding macroevolutionary patterns, but also for understanding microevolution.

Extreme perturbations of the environment are rare and hence difficult to study *in situ*. A prime example of an extreme environmental perturbation is the impact of the meteors in Yucatan during the Cretaceous on climate and subsequent biodiversity on Earth (Alvarez *et al.*, 1980). There are several other similar impacts. Volcanic eruptions such as those of Krakatoa and Mount St. Helens had significant impact on global climate (Delmoral & Bliss, 1993; Zielinski, 1995), and previous volcanic eruptions occasionally had even greater impacts (e.g. de Silva & Zielinski, 1998; Highwood & Stevenson, 2003). Such extreme events may also have had random impacts on the genomes of living organisms via population bottlenecks followed by genetic drift, or the particular

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physiology and ecology of certain species may have produced differences in the ability to cope with such environmental perturbations.

The nuclear disaster at Chernobyl on 26 April 1986 resulted in the release of enormous amounts of radioactive material that contaminated thousands of square kilometres across Europe (e.g. reviews in Shestopalov, 1996; Zakharov & Krysanov, 1996; Møller & Mousseau, 2006; Yablokov *et al.*, 2009). We consider that Chernobyl constitutes an extreme environmental perturbation that may provide important lessons for understanding ecological and evolutionary questions that are otherwise difficult or impossible to study. Chernobyl has significantly increased mutation rates in plants and animals including humans (reviews in Møller & Mousseau, 2006; Yablokov *et al.*, 2009). Severe negative ecological effects such as reductions in abundance and biodiversity of animals including birds have also been documented (Møller & Mousseau, 2007b,c, 2008, 2009). This research suggests ecosystems have been significantly perturbed. The underlying physiological mechanisms accounting for these ecological effects have also been studied. The physiological effects of radioactive contamination include reductions in levels of antioxidants in humans and animals (Bazhan, 1998; Ben-Amotz *et al.*, 1998; Chaialo *et al.*, 1991; Ivaniota *et al.*, 1998; Kumerova *et al.*, 2000; Lykholat & Chernaya, 1999; Neyfakh *et al.*, 1998a,b). Experimental studies involving humans have shown improvements in health associated with beta carotene supplementation (Ben-Amotz *et al.*, 1998). It is also possible that antioxidant levels, as affected by radiation, have had negative effects on mutation rates; several studies have indicated that the presence of high levels of carotenoids reduces mutation rates (reviews in Ferguson, 1994; Krinsky & Denek, 1982; Møller *et al.*, 2000; Sies, 1993; Valko *et al.*, 2004).

The objective of this study was to investigate whether the short-term ecological response to radiation from Chernobyl could be used to predict the accumulation of mutations on an evolutionary time scale. The basis for this objective is that species differ in susceptibility to environmental perturbations (e.g. Hoffmann & Parsons, 1991, 1997), including susceptibility to mutagens or ability to repair DNA (reviews in Hoffmann & Parsons, 1991; Friedberg *et al.*, 2006; Halligan & Keightley, 2009). Likewise, species differ in susceptibility to the impact of radiation from Chernobyl on abundance (Møller & Mousseau, 2007b) and mutation rates (reviews in Kordyum & Sidorenko, 1996; Møller & Mousseau, 2006). Such interspecific differences in susceptibility to environmental perturbations could predict both short-term ecological responses (such as changes in population density) and long-term evolutionary responses (such as DNA base-pair substitutions) if the underlying mechanisms were similar. For example, a specific life history may render certain species particularly susceptible to short-term environmental perturbations, but also to

perturbations over time, with each major perturbation increasing the rate of DNA base-pair substitutions. Alternatively, differences in ecology may render certain species particularly prone to population size fluctuations, with some species showing large fluctuations over time, whereas others show smaller fluctuations over time, and this difference might be expected to generate very different patterns of DNA substitution rates through time (Hartl & Clark, 1997). We tested the prediction that ecological response in terms of reduction in population density as a result of the extreme environmental perturbation caused by radioactive contamination from Chernobyl was related to response to perturbations in the evolutionary past as reflected by DNA base-pair substitution rates. To this end, we used extensive census data of breeding birds collected during 4 years of fieldwork in Ukraine and Belarus (Møller & Mousseau, 2007b, 2008) and an extensive database of DNA base-pair substitution rates in the mitochondrial cytochrome *b* gene (Nabholz *et al.*, 2008, 2009). A second objective was to test whether the underlying mechanism for an association between radiation and abundance and substitution rate, respectively, was based on interspecific differences in availability of antioxidants. To this end, we used an extensive database on concentrations of fat-soluble antioxidants (carotenoids, vitamin E) in the main storage organ, the liver (Møller *et al.*, 2010). We controlled statistically for the potentially confounding effects of two variables: population size and longevity. First, common and widespread species could be better able to cope with adverse environmental conditions, and such species may also fluctuate less in population size than rare species with local distributions. Second, Nabholz *et al.* (2008, 2009) suggested that longevity might be related to substitution rates, with long-lived species being especially affected by exposure to radioactive mutagens. Thus, we tested the extent to which these two variables could account for the association between reduction in population density caused by radiation and DNA base-pair substitution rate.

Materials and methods

Study sites

Anders Pape Møller (APM) (wearing a radiation protection suit in the most contaminated areas) conducted standard point counts during 29 May–9 June 2006, 1–11 June 2007, 29 May–5 June 2008 and 1–6 June 2009. Each count lasted 5 min during which all birds seen or heard were identified and recorded according to standard procedures (Møller, 1983; Bibby *et al.*, 2005). We emphasize that the use of the radiation protection suit did not interfere with the censuses because the hood was not used, allowing the same possibilities for recording birds visually and from calls and songs among sites. For the record, we note that this procedure is fully in

accordance with radiation protection procedures used in the Chernobyl Exclusion Zone. Removal of the < 5% of all census points for which the radiation protection suit was used did not change any of the conclusions reported here. The census was conducted within the Chernobyl Exclusion Zone (or in areas adjacent on the southern and western borders) with a permit from the Ukrainian authorities and in contaminated areas in southern

Belarus around Gomel (breeding seasons 2006–2009) (Fig. 1). A total of 254 points (breeding season 2006), 240 points (breeding season 2007), 237 points (breeding season 2008) and 167 points (breeding season 2009), in total 898 points, were located at ca. 100-m intervals within forested areas [excluding successional stages of secondary forest because of abandoned farming (these areas are still almost exclusively open grassland)]. There

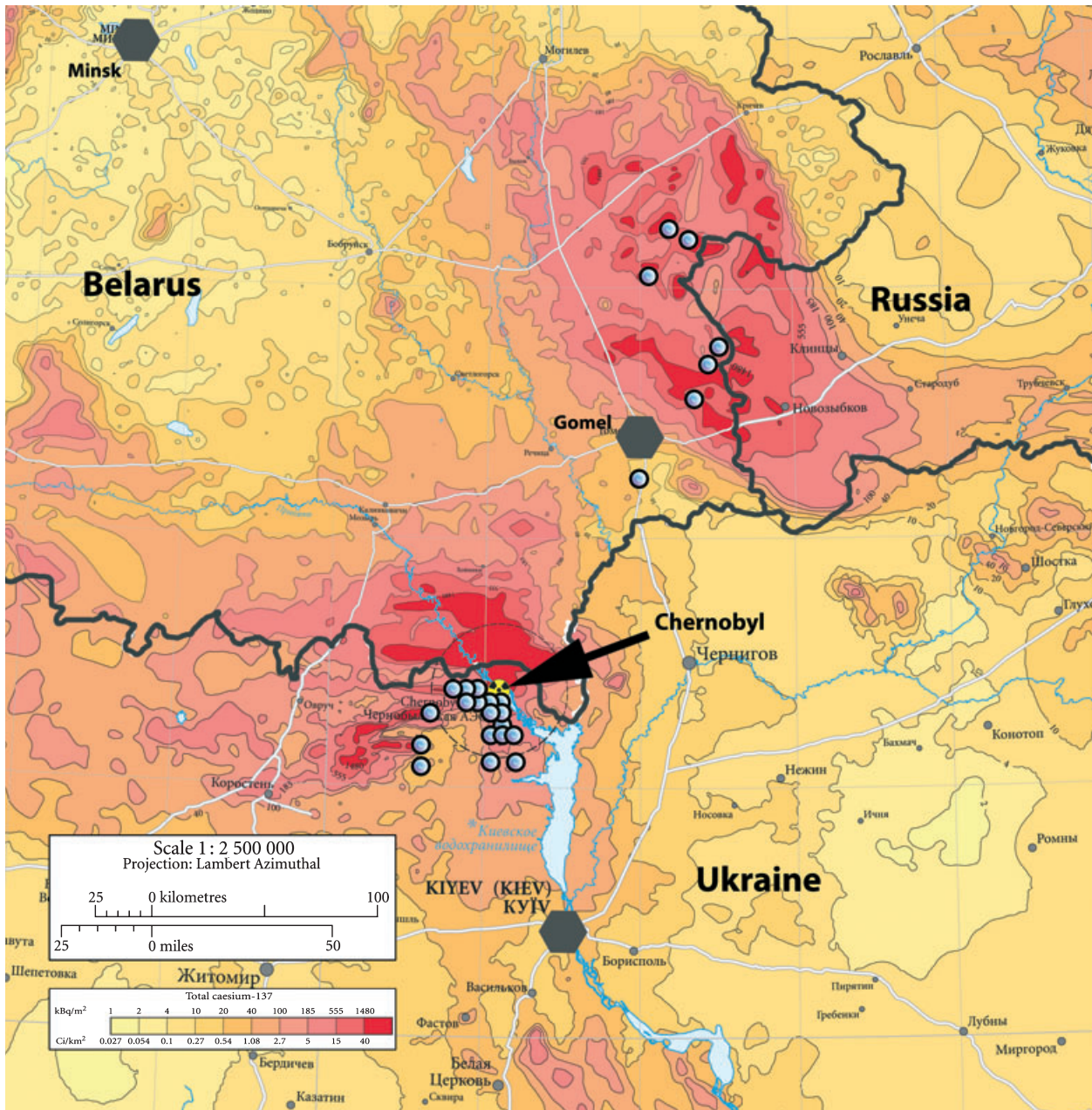


Fig. 1 Location of breeding bird census areas and levels of background radiation around Chernobyl. Partly developed from European Union (1998).

was no temporal and spatial nonindependence of census data because the analyses only relied on the first observation from a given census point, and because highly heterogeneous deposition of radioactive material as a result of the peculiarities of the Chernobyl accident (Møller & Mousseau, 2007a; Shestopalov, 1996; Yablokov *et al.*, 2009 and Fig. 1) have reduced the spatial autocorrelation in background radiation to a negligible level (see data and analyses in Møller & Mousseau, 2007a). In other words, by using neighbouring sites that differ in level of background radiation, we can exclude the possibility that differences in weather, human activity, soil type or any other potentially confounding factor could have biased the analyses.

Bird census data

We censused birds at the end of May and the beginning of June when most individuals reach their annual maximum of singing activity, making censuses of breeding birds highly reliable (Møller, 1983; Bibby *et al.*, 2005). We directly tested the reliability of our counts by letting two persons independently perform counts. The degree of consistency was high for both species richness and abundance (Møller & Mousseau, 2007a). The number of individuals recorded is reported in the Supporting Information.

Confounding habitat and weather variables

Bird abundance estimates can be affected by numerous confounding variables (Møller, 1983; Bibby *et al.*, 2005), and, therefore, it is crucial to control such variables statistically to assess the underlying relationship between radiation and species richness and abundance. We classified habitats (agricultural habitats with grassland or shrub (either currently or previously cultivated), deciduous forest, or coniferous forest) and estimated to the nearest 10% ground coverage by these different habitat types within a distance of 50 m from the observation point. Agricultural habitat included edges between forest and open areas, and the agricultural habitat variable thus also reflected the amount of edge habitat between forest and open areas. Maximum height of trees was estimated to the nearest 5 m, and soil type was recorded as loam/clay or sand. The presence of open water within a distance of 50 m was also recorded. Weather conditions can affect animal activity and hence census results (Møller, 1983; Bibby *et al.*, 2005), and we recorded cloud cover at the start of each point count [to the nearest eighth, range 0–1 during the censuses, mean (SE) = 0.707 (0.011)], temperature [degrees Celsius, range 12–25 °C, mean (SE) = 20.477 (0.102) °C], and wind force [Beaufort, range 0–4 during the censuses, mean (SE) = 2.378 (0.039)]. For each census point, we recorded time of day when the count was started (to the nearest minute). Because bird activity may show a

curvilinear relationship with time of day, with high levels of activity in the morning and to a lesser extent in the evening (Møller, 1983; Bibby *et al.*, 2005), we also included time squared as an explanatory variable.

Measuring background radiation levels

We measured radiation levels in the field and cross-validated these with measurements by the Ministry of Emergencies, Kiev, Ukraine. We measured α , β , and γ radiation at ground level at each census point after having conducted the census (thus making the census blindly with respect to radiation level) using a hand-held dosimeter (Model: Inspector; SE International, Inc., Summertown, TN, USA). We measured levels several (2–3) times at each site and averaged the measurements. Our data were validated with correlation against data from the governmental measurements published by Shestopalov (1996), estimated as the mid-point of the ranges published, with analyses showing a high degree of consistency between methods (Møller & Mousseau, 2007a). Radiation levels vary greatly at a local scale as a result of heterogeneity in deposition of radioactive material after the Chernobyl accident (Fig. 1; Shestopalov, 1996). Our measurements at the census points ranged from 0.01 to 135.89 $\mu\text{Sv h}^{-1}$.

Impact of radiation on population density

We quantified the impact of radiation on population density by relating the number of individuals per census point to \log_{10} -transformed local background radiation levels for each of the 885 census points, while simultaneously controlling statistically for the potentially confounding variables listed above and year as a factor (see Møller & Mousseau (2007b) for further details). The slope of the partial effect of radiation on abundance was subsequently used as an estimate of the effect of radiation on local population density. Slope estimates were highly consistent among years (Møller & Mousseau, 2010), showing that we could obtain similar information about the impact of radiation on abundance in different years.

Population size

Population sizes were obtained from Hagemeyer & Blair (1997), who reported the total number of breeding pairs in the Western Palearctic west of the Ural Mountains, obtained in a consistent way from national bird census programmes in all countries. We used the arithmetic mean of the minimum and maximum estimates.

Range size

We determined the global northernmost and southernmost latitude of the breeding and the wintering distributions, respectively, to the nearest tenth of a degree of

all species. Breeding range was the northernmost minus the southernmost breeding latitude, whereas wintering range was the northernmost minus the southernmost winter latitude. Information on breeding and wintering ranges was obtained from Cramp & Perrins (1988–1994). These estimates have previously been shown to provide biologically meaningful measures of distribution (Gaston & Blackburn, 1996).

We also estimated breeding range in the Western Palearctic and total breeding range as the area of the shape bounded by the greatest span of latitude and longitude of each species' breeding range, as published in Cramp & Perrins (1988–1994). To take account of the curvature of the earth (which was assumed to be spherical), this area was estimated by the equation

$$\text{Area} = R^2 \times (\text{Longitude}_1 - \text{Longitude}_2) \times (\sin(\text{Latitude}_1) - \sin(\text{Latitude}_2))$$

where R are the radius of the earth (6366.2 km), and latitude and longitude are expressed in radians.

In widespread species, Old and New World ranges were calculated separately and subsequently summed. This method overestimates true geographical range because parts of the range that are unoccupied are also included, although this error should be random with respect to the variables of interest. Estimates of area were strongly positively correlated with geographical range size as calculated by counting one-degree grid cells overlain on published distribution maps for a sample of 20 Palearctic and Nearctic bird species ($r = 0.87$, $P < 0.001$), and with range size as reported for a sample of 11 threatened species (Stattersfield & Capper, 2000) ($r = 0.98$, $P < 0.001$, based on log-transformed data). See Møller *et al.* (2008) for further information.

Longevity

We estimated longevity using records of ringed birds adjusted for sampling effort; this approach has been proven to provide unbiased estimates (Møller, 2006). We extracted information on maximum longevity of European species from EURING. Longevity records only provide reliable information on maximum lifespan if records are adjusted for sampling effort. Among the 120 species of common birds in Europe for which longevity records were available for the present study, the total number of recoveries and recaptures of banded birds across Europe ranged from 110 to 187 764, with a total of 1 953 714 records (<http://www.euring.org>). Therefore, we used total number of recoveries reported as a measure of variation in sampling effort.

Mitochondrial DNA substitution rates

Species-specific mtDNA base-pair substitution rates were used as reported by Nabholz *et al.* (2008, 2009). Briefly,

complete cytochrome *b* sequences for 1571 avian species were aligned, and changes in the third base-pair of amino acid encoding codons were used as a measure of genetic divergence (Nabholz *et al.*, 2009). Such changes in the third base-pair of amino acid encoding codons are most often synonymous with respect to amino acid changes and are thus not usually under selection. The third codon position substitution rate provides a good approximation of synonymous substitution rate for two different reasons. First, the vast majority of the cytochrome *b* divergence is synonymous (the ratio nonsynonymous/synonymous divergence is close to 2% (Nabholz *et al.*, 2008; Stanley & Harrison, 1999). Second, all the transition substitutions (A<->G and T<->C) of the cytochrome *b* third codon position are synonymous, and most of the substitution are transitions (the ratio transition/transversion being typical > 15 in the dataset).

Antioxidants in liver

JE received 660 specimens for taxidermy from Denmark, with most individuals originating from a radius of 100 km from Christiansfeld, Denmark, and he collected a sample of fresh liver for biochemical analyses. Livers were frozen immediately after collection and maintained at $-20\text{ }^{\circ}\text{C}$ until analysis. Any livers that were not absolutely fresh were discarded from the present study. For all specimens, JE also recorded date, year, site and cause of death upon receipt.

Vitamin E concentrations were determined using a Shimadzu Prominence (Kyoto, Japan) full high performance liquid chromatography (HPLC) system (Sil-20A Autosampler; LC-20AD solvent delivery system; RF-10 A_{XL} Spectrofluorometric detector, CBM-20Alite system controller; Cto-100ASvp column oven) fitted with a Spherisorb, type S30DS2, 3 μm C-18 reverse phase HPLC column (15 cm \times 4.6 mm; Phase Separations, Cambridge, UK). Chromatography was performed using a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1.05 mL min⁻¹. Fluorescence detection of vitamin E used excitation at 295 nm and emission at 330 nm. Peaks of δ -, γ - and α -tocopherol were identified by comparison with the retention time of standards of tocopherols (Sigma, Poole, UK). All sampled livers were analysed for vitamin E concentration. Vitamin E was calculated as the summed concentrations of δ -, γ - and α -tocopherol. Concentrations and not quantity of vitamin E were used as the variable of interest in statistical analysis because concentration is the main factor in determining physiological action of antioxidants at the level of tissues (Surai, 2002). The inter-assay coefficient of variation for α -tocopherol determination was 3.9% (Surai *et al.*, 1999).

Total carotenoid concentration of liver was determined using the same HPLC system with a diode array detector at 444 nm, fitted with a Waters Spherisorb type NH2 column (25 cm \times 4.6 mm; Phase Separation) with a mobile phase

of methanol-distilled water (97 : 3), at a flow rate of 1.5 L min⁻¹ as described by Hõrak *et al.* (2002). The HPLC was calibrated using lutein standards (Sigma). All analytic detections were performed at 30 °C in column oven and a constant heating, ventilating and air conditioning (HVAC) controlled room temperature of 24 °C.

Estimates of total carotenoids and vitamin E were significantly correlated among individuals of the same species [carotenoids: $F_{152,506} = 4.03$, $P < 0.0001$, R (SE) = 0.37 (0.03); vitamin E: $F_{152,506} = 1.65$, $P < 0.0001$, R (SE) = 0.10 (0.02)]. Møller *et al.* (2010) provide a full description of this dataset, but also show that this dataset contains biologically relevant information.

Body mass

We recorded body mass from Cramp & Perrins (1988–1994), using information from the breeding season and preferably the estimate with largest sample size, if more than a single estimate was available. All data are reported in Appendix S1.

Statistical analyses

Radiation level, substitution rate, concentration of carotenoids and vitamin E and body mass were log₁₀-transformed, whereas ground coverage with farmland and deciduous forest was square root arcsine-transformed (coniferous forest was not included as an explanatory variable, as it simply represented the ground coverage not attributed to farmland and deciduous forest) to achieve approximately normal distributions. None of the distributions deviated significantly from normal distributions after transformation. We also included radiation level squared to account for nonlinear relationships between species richness and abundance, respectively, and radiation. We developed statistical models to assess the relationship between species richness and abundance (response variables) and radiation (predictor variable), assuming a Poisson distribution, after inclusion of the potentially confounding variables (additional predictor variables listed above), as implemented in the statistical software JMP (SAS Institute Inc., 2000).

We quantified the relationship between abundance of different species and level of radiation by calculating the slope of the relationship between number of individuals per census point and log₁₀-transformed radiation for each species. These slopes that are statistically heterogeneous among species (Møller & Mousseau, 2007a) were used for subsequent analyses, with each slope being weighted by sample size to account for unequal intensity of sampling.

We related the substitution rate to the slope of the relationship between abundance and radiation. Because sample sizes differed considerably among species owing to differences in abundance, we conducted a second

series of analyses that weighted estimates by sample size. In order to avoid giving a very large weight to the most abundant species, we used square root-transformed sample size as a weight (Garamszegi & Møller, 2010). Analyses using weights based on square root of sample sizes, log₁₀-transformed sample sizes or the standard error of the slopes gave qualitatively similar conclusions.

Comparative analyses

Closely related species have more similar phenotypes than species that are more distantly related, simply because similarity among closely related species is likely to be because of to such species sharing a recent common ancestor. We controlled for similarity in phenotype among species owing to common ancestry by calculating standardized independent linear contrasts (Felsenstein, 1985), using the program CAIC (Purvis & Rambaut, 1995). We tested the statistical and evolutionary assumptions of the continuous comparative procedure (Garland *et al.*, 1992) by regressing absolute standardized contrasts against their standard deviations. To reduce the consequent problem of heterogeneity of variance, (i) outliers (contrasts with Studentized residuals > 3) were excluded from subsequent analyses (Jones & Purvis, 1997), and (ii) analyses were repeated with the independent variable expressed in ranks. In neither case did these new analyses change any of the conclusions.

The composite phylogeny used in the analyses was mainly based on Sibley & Ahlquist (1990), combined with information from more recent sources (Hackett *et al.*, 2008; Jönsson & Fjeldså, 2006; Appendix S2). Because information for the composite phylogeny came from different studies using different methods, consistent estimates of branch lengths were unavailable. Therefore, branch lengths were transformed assuming a gradual model of evolution with branch lengths being proportional to the number of species contained within a clade. Results based on these branch lengths were compared to those obtained using constant branch lengths (a punctuated model of evolution). Nowhere were results qualitatively different.

Regressions based on contrasts were forced through the origin since the comparative analyses assume that there has been no evolutionary change in a character when the predictor variable has not changed (Purvis & Rambaut, 1995).

We assessed relationships based on effect sizes according to the criteria listed by Cohen (1988) for small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate (9% of the variance) or large effects (25% of the variance).

Results

The combined abundance of all species of birds at census points was negatively related to background radiation

[$F_{1,28672} = 28.52$, $P < 0.0001$, slope (SE) = -0.0018 (0.0003)], it differed among species ($F_{31,28672} = 134.00$, $P < 0.0001$), and the effect of radiation differed among species ($F_{31,28672} = 13.83$, $P < 0.0001$). Eleven of these slopes were statistically significant, 10 negative and one positive. Because there were significant differences in slope among species, it is meaningful to investigate predictors of this heterogeneity. The substitution rate in the sample of 32 species studied was on average 0.0480 changes in the third base-pair of amino acid encoding codons for cytochrome *b* per site per million years (SE = 0.0072), range 0.0067–0.2020, differing significantly from zero (one-sample *t*-test, $t_{31} = 6.66$, $P < 0.0001$).

Species more strongly affected by radiation had higher substitution rates (Fig. 2), accounting for 17% of the variance (Table 1), implying that mutation rates were higher in species that were more strongly negatively affected by radiation. Bootstrapped estimates of the slope were -0.0303 (0.0124), based on 1000 bootstraps, almost identical to the estimate reported in Table 1. Because the slope estimate was based on highly variable sample sizes owing to differences in abundance, we used a second model that weighted species-specific observations by square root-transformed sample size. This model was again highly significant, explaining 32% of the variance (Table 1). Using log-transformed sample size as a weighting factor provided an equally strong relationship [$F_{1,30} = 10.44$, $P = 0.0030$, slope (SE) = -0.051 (0.016)]. Likewise, weighting by the standard error of the slope estimate provided a qualitatively similar conclusion [$F_{1,30} = 18.03$, $P = 0.0002$, slope (SE) = -0.064 (0.015)]. Inclusion of body mass as an additional predictor variable significantly improved the fit of the model [species-specific analyses: substitution rate partial $F_{1,29} = 14.25$, $P = 0.0007$, slope (SE) = -0.078 (0.021),

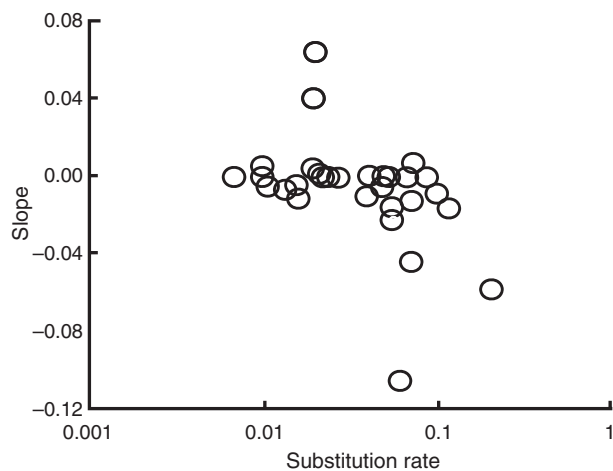


Fig. 2 Slope of the relationship between population density of different species of breeding birds and radiation level around Chernobyl in relation to mitochondrial substitution rate.

Table 1 Models relating slope of the relationship between population density of different species of birds and radiation around Chernobyl and mitochondrial DNA substitution rate.

| Variable | Sum of squares | d.f. | F | P | Slope (SE) |
|---|----------------|------|-------|--------|------------------|
| Species | | | | | |
| Mitochondrial DNA substitution rate | 0.711 | 1 | 6.19 | 0.019 | -0.030 (0.012) |
| Error | 3.446 | 30 | | | |
| Mitochondrial DNA substitution rate weighted by sample size | 0.089 | 1 | 13.97 | 0.0008 | -0.069 (0.018) |
| Error | 0.191 | 30 | | | |
| Contrasts | | | | | |
| Mitochondrial DNA substitution rate | 0.00066 | 1 | 4.19 | 0.048 | -0.031 (0.014) |
| Error | 0.0049 | 30 | | | |
| Mitochondrial DNA substitution rate weighted by sample size | 0.0044 | 1 | 5.59 | 0.025 | -0.036 (0.015) |
| Error | 0.0220 | 30 | | | |

with an additional effect of body mass: partial $F_{1,29} = 0.092$, $P = 0.035$, slope (SE) = -0.014 (0.015); contrasts: substitution rate partial $F_{1,29} = 5.87$, $P = 0.022$, slope (SE) = -0.051 (0.021); body mass partial $F_{1,29} = 0.02$, $P = 0.89$, slope (SE) = -0.002 (0.016)].

Two species had extreme slope values (Fig. 2), suggesting that a nonparametric analysis might be more appropriate. A nonparametric Kendall rank-order correlation also revealed a highly significant negative relationship (Kendall $\tau = -0.30$, $P = 0.018$). Even exclusion of these two species produced a highly significant negative relationship accounting for 42% of the variance [weighted analysis: $F_{1,28} = 18.51$, $r^2 = 0.40$, $P = 0.0002$, slope (SE) = -0.042 (0.010)].

Because previous studies have indicated that bird species have reduced carotenoid levels in more contaminated areas, and that species with carotenoid-based plumage have more suppressed population densities, we analysed the independent effects of carotenoid levels and substitution rates on slopes. This analysis weighted by sample size showed a statistically significant effect of substitution rate, but not of carotenoids on the slope (Table 2). Likewise, the analysis based on linear contrasts showed a significant effect for substitution rate, but not for carotenoids (Table 2). Vitamin E did not enter any of these models as a significant predictor.

We developed a statistical model with slope of the relationship between abundance and background radiation level as the response variable and substitution rate and population size as predictors. Only the former predictor showed a significant effect [species-specific analysis weighted by sample size: partial $F_{1,29} = 13.90$, $r^2 = 0.32$, $P = 0.0008$, slope (SE) = -0.071 (0.019)],

Table 2 Models relating slope of the relationship between population density of different species of birds to radiation around Chernobyl and mitochondrial DNA substitution rate and carotenoid levels in the liver, weighted by square root-transformed sample sizes.

| Variable | Sum of squares | d.f. | F | P | Slope (SE) |
|---|----------------|------|-------|--------|----------------|
| Species | | | | | |
| Mitochondrial DNA substitution rate weighted by sample size | 0.112 | 1 | 12.51 | 0.0024 | -0.103 (0.029) |
| Carotenoids | 0.016 | 1 | 1.79 | 0.20 | -0.028 (0.021) |
| Error | 0.161 | 18 | | | |
| Contrasts | | | | | |
| Mitochondrial DNA substitution rate weighted by sample size | 0.010 | 1 | 6.95 | 0.017 | -0.064 (0.024) |
| Carotenoids | 0.002 | 1 | 1.23 | 0.28 | -0.016 (0.015) |
| Error | 0.026 | 18 | | | |

The model had the statistics $F_{2,18} = 6.57$, $r^2 = 0.42$, $P = 0.007$ and $F_{1,18} = 3.63$, $r^2 = 0.17$, $P = 0.047$.

whereas that was not the case for population size [partial $F_{1,29} = 0.28$, $r^2 = 0.01$, $P = 0.60$, slope (SE) = 0.005 (0.010); contrast analysis weighted by sample size: partial $F_{1,29} = 5.93$, $r^2 = 0.17$, $P = 0.021$, slope (SE) = -0.049 (0.020)], but not for population size [partial $F_{1,29} = 1.35$, $r^2 = 0.04$, $P = 0.25$, slope (SE) = -0.011 (0.009)]. A similar conclusion was reached if range size was included as an additional variable to account for the fact that species with larger ranges obviously have larger population sizes [species-specific analysis weighted by sample size: substitution rate partial $F_{1,28} = 14.97$, $r^2 = 0.35$, $P = 0.0006$, slope (SE) = -0.070 (0.018); population size partial $F_{1,28} = 1.99$, $r^2 = 0.07$, $P = 0.17$, slope (SE) = 0.016 (0.011); range size partial $F_{1,28} = 3.42$, $r^2 = 0.11$, $P = 0.08$, slope (SE) = -0.157 (0.085); contrast analysis weighted by sample size: substitution rate partial $F_{1,28} = 5.64$, $r^2 = 0.17$, $P = 0.025$, slope (SE) = -0.046 (0.019); population size partial $F_{1,28} = 0.10$, $r^2 = 0.00$, $P = 0.75$, slope (SE) = 0.004 (0.012); range size partial $F_{1,28} = 3.25$, $r^2 = 0.10$, $P = 0.08$, slope (SE) = -0.120 (0.066)].

In a final series of analyses, we tested for an effect of longevity, with sample size for longevity as an additional variable. There was still a strong relationship between slope of the relationship between abundance and background radiation level as the response variable and substitution rate, longevity and sample size for longevity as predictors, with only the former showing a significant effect [species-specific analysis weighted by sample size substitution rate partial $F_{1,25} = 11.97$, $r^2 = 0.32$, $P = 0.0019$, slope (SE) = -0.075 (0.022); contrast analysis weighted by sample size substitution rate partial $F_{1,25} = 5.01$, $r^2 = 0.17$, $P = 0.034$, slope (SE) = -0.041 (0.018)], but not for longevity [species-specific analysis

weighted by sample size partial $F_{1,25} = 0.36$, $r^2 = 0.01$, $P = 0.55$, slope (SE) = -0.030 (0.050); contrast analysis weighted by sample size partial $F_{1,25} = 0.32$, $r^2 = 0.01$, $P = 0.58$, slope (SE) = 0.017 (0.030)], or number of recoveries [species-specific analysis weighted by sample size partial $F_{1,25} = 0.83$, $r^2 = 0.03$, $P = 0.37$, slope (SE) = -0.009 (0.010); contrast analysis weighted by sample size partial $F_{1,25} = 2.51$, $r^2 = 0.09$, $P = 0.13$, slope (SE) = -0.010 (0.006)].

Discussion

Here, we have shown that species of birds that have severely reduced population densities in contaminated areas are the same species that have accumulated the most mutations over time as reflected by third base-pair substitutions in codons of cytochrome *b*. We investigated a number of alternative explanations, but found no evidence that any of these factors accounted for the main result. This major finding is consistent with our hypothesis. Body size is an important predictor of substitution rate (reviews in Nabholz *et al.*, 2008, 2009), but the effect of body size did not confound any of these conclusions. These effects were similar when the analyses were adjusted for interspecific differences in sampling effort. Likewise, comparative analyses investigating the relationship after taking similarity among species owing to common phylogenetic descent into account resulted in similar conclusions. Because levels of antioxidants are significantly suppressed in birds in the most contaminated areas, we conducted a second series of analyses relating substitution rates to both impact of radiation on abundance and antioxidant levels. However, the relationship between substitution rate and impact of radiation on abundance was independent of concentrations of antioxidants. Finally, effects of population size and longevity did not confound the conclusions, nor were such effects to be expected for a neutral genetic marker. We interpret these findings as being consistent with our hypothesis that species differ in susceptibility to extreme environmental perturbations, and that such differences are reflected both on a short time scale as shown for abundance and radiation, but also on a long time scale as for substitution rates. The underlying hypothetical mechanism is that species differ in their ability to repair DNA, which affects both DNA substitution rates and susceptibility to radiation from Chernobyl.

Radiation from environmental contamination as a result of the Chernobyl explosion has had important negative effects on mutations, abundance and physiology of animals and plants (reviews in Zakharov & Krysanov, 1996; Møller & Mousseau, 2006; Yablokov *et al.*, 2009). In the present study, we investigated the impact of radiation on abundance of common breeding birds around Chernobyl in Ukraine and Belarus and related the impact of radiation on abundance to a

measure of historical mutation rates as reflected by neutral third base-pair substitutions in codons of cytochrome *b*. The basis for this hypothesis is that species differ in susceptibility to environmental perturbations, as shown by extensive ecological and physiological studies (e.g. Hoffmann & Parsons, 1991, 1997; Bokony *et al.*, 2009). Likewise, both plants and animals differ in susceptibility to mutagens or efficiency of DNA repair (reviews in Hoffmann & Parsons, 1991; Friedberg *et al.*, 2006; Halligan & Keightley, 2009). Similar effects have been reported based on research at Chernobyl. Species of plants and animals differ in susceptibility to the impact of radiation from Chernobyl on abundance (Møller & Mousseau, 2007b) and mutation rates (reviews in Kordyum & Sidorenko, 1996; Møller & Mousseau, 2006). Such interspecific differences in susceptibility could predict both short-term ecological responses (such as changes in population density) and long-term evolutionary responses (such as DNA base-pair substitutions) if the underlying mechanisms were similar. Here, we have shown that species of birds that have severely reduced population densities in contaminated areas are the same species that have accumulated the most mutations over time as reflected by third base-pair substitutions in codons of cytochrome *b*. We investigated a number of alternative explanations, but found no evidence that any of these factors accounted for the main result. This major finding is consistent with our hypothesis.

There was a significant negative relationship between the effects of background radiation on abundance and third base-pair substitutions in codons of cytochrome *b* with an intermediate to large effect size (*sensu* Cohen, 1988), implying that mutation rates were higher in species that were more adversely affected by radiation. This conclusion remained unchanged by a number of alternative analyses, exclusion of extreme data points, and analyses corrected for possible phylogenetic biases. Many factors are known to influence bird census results (Møller, 1983; Bibby *et al.*, 2005), and we included these potentially confounding factors in the statistical analyses. Obviously, we cannot be certain that some as yet unknown confounding factor was included, although this seems unlikely given the fact that standardized national breeding bird census programmes have been conducted in Europe since the 1960s, and that such programmes are implemented in environmental monitoring programmes at national and continental scales. These surprising findings imply that ecological effects of radiation, as reflected by the reduced abundance of birds in radioactively contaminated areas, provide important information on the ability of individuals of different species to cope with environmental perturbations in the past. Numerous large environmental impacts have occurred on an evolutionary time scale, affecting the distribution and abundance of taxa, but also their extinction rates (e.g. Lawton & May, 1995). Mass

extinctions such as those during the Permian (e.g. Bowring *et al.*, 1998; Raup & Sepkoski, 1982) and the boundary between the Cretaceous and the Tertiary have been linked to meteor impacts on Earth (e.g. Alvarez *et al.*, 1980; Jablonski, 1986). Here, we have shown that long-term substitution rates during evolutionary time can be used to make inferences about the impact of extreme environmental perturbations during ecological time. This also implies that responses of living organisms to extreme perturbations such as the Chernobyl disaster can have important repercussions for basic evolutionary and conservation research.

We investigated the underlying physiological mechanisms by analysing the relationship between antioxidants and third base-pair substitutions in codons of cytochrome *b*. Studies of humans (Bazhan, 1998; Ben-Amotz *et al.*, 1998; Chaihalo *et al.*, 1991; Ivaniota *et al.*, 1998; Kumerova *et al.*, 2000; Lykholat & Chernaya, 1999; Neyfakh *et al.*, 1998a,b) and animals (Møller & Mousseau, 2007b; Møller *et al.*, 2005, 2008) have shown severely reduced levels of antioxidants in individuals exposed to radiation, with effects being described for carotenoids and vitamin E. Here, we found that bird species with strong impacts of radiation on abundance also had reduced levels of carotenoids stored in the liver, but not of vitamin E, independent of the relationship for substitution rates. However, this apparent effect of carotenoids was not maintained following corrections for phylogenetic biases, suggesting that the effect of carotenoids could not have confounded the relationship between substitution rate and effect of radiation on abundance.

Finally, we investigated the potential confounding effects of population size and longevity on the conclusions, but found no evidence consistent with these hypothetical effects. Thus, the conclusions were robust and remained unaffected by any of the obvious potentially confounding variables.

In conclusion, we have found that neutral mutations in cytochrome *b* accumulated during evolutionary time were a predictor of the response of population density of birds to exposure to elevated background radiation from Chernobyl.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Summary statistics of DNA substitution rate (divergence per site per million years), the slope of abundance of different species of birds in relation to local radiation level, after controlling statistically for confounding variables affecting abundance estimates, number of individuals censused at Chernobyl, population size in Western Palearctic, range size in Western Palearctic (km²), longevity record (years), sample size for longevity record, concentration of carotenoids and vitamin E in the liver ($\mu\text{g g}^{-1}$) and body mass.

Appendix S2 Phylogenetic relationships among species.

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