

# Avian genetic ecotoxicology: DNA of the canary in a coalmine

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**Abstract** Genotoxic chemicals, through damage and alteration of the genetic material of wild organisms, pose significant threats to the persistence of wild animal populations. Their damaging effects can ultimately impair the health of the ecosystem and its provision of services to human society. Bird species are good candidates for the role of sentinels of the effects of genotoxins, thanks to (i) the diversity of their ecological niches, (ii) their ubiquity across environments, (iii) their conspicuousness, abundance and approachability, together with (iv) their well-known life histories and the availability of historical data series. Avian diversity increases the likelihood that adequate model species be available for monitoring genotoxicants and assessing their impact. This paper reviews the methods utilized by genetic ecotoxicological studies of wild birds, highlighting their benefits and shortcomings. It also summarizes the genetic ecotoxicological studies so far conducted. In spite of a paucity of studies, several classes of genotoxicants have already been investigated across a variety of species and environments, thus supporting the versatility of birds as monitors of genotoxic contamination. Future technical advancements and applications are suggested, with particular reference to the analysis of mutational events, gene expression and methylation patterns. Finally, I argue that the development of avian genetic ecotoxicology will contribute to the understanding of natural variation in the underlying machinery for coping with DNA damage and oxidative stress, both of which are increasingly recognized as proximate factors in the evolution of life history adaptations [Current Zoology 60 (2): 285–298, 2014].

**Keywords** Biomarker, DNA damage, Ecogenotoxicology, Ecological indicator, Ecological Risk assessment, Environmental monitoring

“You live your life like a canary in a coalmine  
You get so dizzy even walking in a straight line”  
Police, Canary in a coalmine, 1980

## 1 Introduction

Genetic ecotoxicology, or ecogenotoxicology, is the study of the damages inflicted by contaminants to the genetic material of wild populations of plants and animals (Kleijnans and van Schooten, 2002; Shugart and Theodorakis, 1994). Its ultimate goal is to assess the threat that such contaminants pose to individual fitness, as well as to the persistence of natural populations (Anderson and Wild, 1994; Depledge, 1994).

The techniques employed in genetic ecotoxicology can be classified in two main categories, according to whether the main aim is to monitor the distribution of the genotoxicant or its effects in the biological systems (Kleijnans and van Schooten, 2002). The first aim is equivalent to estimating the dose of the contaminant that impacted the genetic material (Shugart, 2000). The evaluation of the biological response, instead, can range from the analysis of the genetic and cytological response to its implications for other physiological and

behavioral functions, and ultimately for population-level consequences and other ecological dynamics (Anderson et al., 1994; Jha, 2008; Relyea and Hoverman, 2006).

The studies on the ecological fate of genotoxic agents have traditionally focused on aquatic species, largely because the lipophilic behavior of genotoxins causes their accumulation in the living compartment of the aquatic ecosystem (Jha, 2004). Yet, genotoxic chemicals can also be airborne or move throughout the terrestrial ecosystem (Somers et al., 2002).

In this review, I argue that bird species are good candidates as sentinel organisms in genetic ecotoxicological studies thanks to (i) the high diversity of ecological niches that they occupy, (ii) their ubiquity across environments, and (iii) their abundance, conspicuousness and relative approachability. By virtue of all these characteristics, a wealth of information exists concerning their behavior and life history, which reflects the large contribution of bird studies to the understanding of the

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natural world, particularly in the field of behavioral and evolutionary ecology (Konishi et al., 1989). Such availability of information can inform the choice of the species and provide reference points for monitoring the dispersal and the effects of contaminants. In fact, the arguments in support of using bird species in ecogenotoxicological studies are not different from those that have traditionally been put forward to promote the more general use of birds as biomonitors of environmental contamination (Bibby, 1999; Burger and Gochfeld, 2004; Carere et al., 2010; Furness, 1993; Furness and Camphuysen, 1997; Goodale et al., 2008; Newman et al., 2007; Furness and Camphuysen, 1997). Ecotoxicologists have long relied on birds for assessing the distribution and effects of contaminants, irrespective of their effects on the genetic material, using destructive and non-destructive sampling of a variety of tissues, and assessing the concentration of contaminants as different as heavy metals, pesticides, halogenated aromatic hydrocarbons (HAH), polychlorinated biphenyls (PCBs), and many others (Furness, 1993). The best known example of bird species acting as an early sentinel of ecosystem-wide toxicological risk is perhaps the bioaccumulation of the dichlorodiphenyltrichloroethane (DDT), which infamously caused thinning of eggshells and consequent reproductive failure and populations crashes of bird species, particularly so for top predators (Hellou et al., 2012).

Traditionally, birds have also been used to monitor ecological processes of socio-economic interest, such as food web structure and the dynamics of fish stocks (Cury et al., 2011; Iverson et al., 2007; Springer et al., 2007), ecosystem health (Newman et al., 2007; Smits and Fernie, 2013), forest and agricultural management (Benton et al., 2002), and the response of biological systems to climate change (Møller et al., 2008; Springer et al., 2007; Sydeman et al., 2012). In a few occasions, the aforementioned benefit of using well known, approachable species allowed a remarkably precise identification of the physiological mechanism linking population-level response to the ecological process under study (Kitaysky et al., 2006). In a similar fashion, species composition of a bird community can also be used to infer anthropogenic disturbance to the ecosystem, and its likely effects on other taxa (O'Connell et al., 2000). Perhaps, there is no group of birds in which this potential 'biomonitoring role' has been emphasized more than in seabirds, which have been widely used for assessing pollution in coastal and marine environments (Burger

and Gochfeld, 2004; Furness and Camphuysen, 1997). The advantages of using seabirds as biomonitors of pollution include their conspicuousness and their longevity, the latter of which allows for long, integrated exposure to pollutants, and potentially for longitudinal sampling of the same individuals over time. Importantly, many seabirds are also charismatic species, which makes it easier to fund monitoring programs and engage the general public (Burger and Gochfeld, 2004).

Most of the studies that used birds as monitors of environmental status and change, however, have not coupled their assessment with the use of any biomarker of genotoxicity. Yet this investigation has been performed in a few different bird species, for contaminants that included lead from ammunition dispersed in the environment (Fisher et al., 2006), persistent organic pollutants (Custer et al., 2007; 2000; Skarphedinsdottir et al., 2010), airborne traffic-related chemicals (Schilderman et al., 1997; Siculo et al., 2010), toxic spills (Pastor et al., 2001b; 2004), and radioactive contamination (Bonisoli Alquati et al., 2010; Ilyinskikh et al., 1997). In another instance, the detection of higher DNA damage in the peripheral blood of Royal terns *Sterna maxima* even led to the postulation of a previously undetected genotoxicant (Maness and Emslie, 2001).

The diversity of the chemical compounds that have been analyzed in these (admittedly few) studies supports the idea that birds are good sentinels for ecogenotoxicological studies. In fact, birds are good candidates for biomonitoring the distribution and effects of genotoxins for several reasons. First, the diversity of ecological roles and niches that birds occupy in any ecosystem implies that they feed on a variety of food sources, making it virtually certain that some of them will be exposed to the contaminant under investigation. Conversely, the measurement of high levels of a genotoxicant in biological tissues or the detection of a genotoxic response can allow the inference on the distribution of the contaminant at lower trophic levels. In fact, many bird species occupy top-level positions in many food webs, allowing for biomagnification of the genotoxicants (Hellou et al., 2012), which makes them more easily detectable. In addition, the high diversity of developmental modes (along the spectrum from altricial to precocial) (Starck, 1998) and socio-sexual systems configure a variety of targets and paths of exposure to genotoxicants.

Secondly, not only birds occupy a variety of niches within a given ecosystem, they also are geographically

ubiquitous. Finally, particularly when compared with mammals, birds are more abundant and conspicuous, and can be more easily approached and captured. Standard, inexpensive census techniques can be easily applied to evaluate their abundance and their species diversity (Bibby 1999; 2004), thus testing for the translation of genotoxic insults into population and community-level effects.

This review has three aims: (i) reviewing the genotoxicological assays and the scope for their application to bird species; (ii) reviewing the genetic ecotoxicological studies so far conducted on avian species; (iii) discussing the benefits of such application for environmental monitoring, as well as for improving our understanding of ecological and life history variation.

Importantly, variation across species in both the genotoxic effect and the early genotoxic response can help predict the ecological fate of species in the wake of ecological disturbance, whether anthropogenic or not.

A better understanding of avian genetic toxicology will inform the choice of species that applied scientists should focus upon while assessing the impact of human activities and habitat degradation. The expansion of the knowledge of intra- and interspecific variation in susceptibility of birds to genetic damage will also facilitate the study of the proximate bases of life history variation within as well as among species. The fields that can be impacted include sexual selection, the evolution of life history, and the physiological bases of resilience to environmental change.

## 2 Ecogenotoxicological Assays and Biomarkers

In this review, the term ‘biomarker’ is referred to as a biological endpoint induced by a xenobiotic at the biochemical, cellular, histological or physiological level (Fossi, 1994; Van Gestel and Van Brummelen, 1996). Importantly, in accordance with van Gestel and van Brummelen (1996), I stress that the term ‘biomarker’ should be kept distinct from the related concept of ‘bioindicator’, which refers to a higher level of biological complexity, i.e. at the organism level or above (Van Gestel and Van Brummelen 1996). In the context of ecogenotoxicology, a ‘bioindicator’ is an individual or a species that, by virtue of its behavior, presence/absence or abundance, can reliably testify on exposure to a genotoxicant and/or on the onset of other stressors. Ideally, variation at the organismal and population levels should match a similar response at the mechanistic level of the ‘biomarkers’ i.e. biochemical, cellular, etc

(Anderson et al., 1994; Forbes et al., 2006). In ecogenotoxicology, a good bioindicator is ‘a canary in a coalmine’, whose silence loudly signals the damage to its DNA. For a bioindicator to act as an efficient sentinel, the effect of exposure on individual fitness and population processes should arise early, before any impact on human and environmental health. In other words, one of the requirements of a reliable bioindicator is to provide ‘early warning’, to be explored through a higher-tier assessment, even at a cost of a ‘false alarm’ for human health (Forbes et al., 2006). Thus, throughout this paper the term ‘sentinel’ is used to refer to the capacity of a species to incorporate and signal information related to the distribution and effect of a contaminant early after the release of the contaminant in the environment. This is a slightly different meaning than the suggestion that a sentinel species should incorporate in its tissue the contaminant under investigation *without* experiencing any deleterious effect (Beeby, 2001).

Biomarkers for genetic ecotoxicology can be classified in two broad categories: those that aim at monitoring and quantifying exposure to the genotoxicant, sometimes referred to as ‘biomarkers of exposure’ (Kleinjans and van Schooten, 2002), and those that characterize the biological response to the agent itself, following the initial interaction of the genotoxicant with DNA (‘biomarkers of effect’). Endpoints measuring DNA damage typically fall in the category of ‘biomarkers of exposure’, as they aim to estimate the exposure to the contaminant through the assessment of a physical or chemical modification of the genetic material. Downstream to this interaction are the biomarkers of the genetic response to the contaminant, including changes in gene expression and gene products, and the induction of the cellular machinery for DNA repair.

In fact, most of these markers were initially developed in the biomedical field, particularly to monitor cancer risk and progression. In the context of ecogenotoxicological studies, their relationship with cancer etiology is not the focus. It should be stressed, however, that there is increasing interest in the ecological and evolutionary significance of cancer for wildlife (Vittecoq et al., 2013). Hence, the development of biomarkers of genotoxicity could become useful for future investigation of oncogenic phenomena in wild populations (Vittecoq et al., 2013).

The biomarkers most frequently used in genetic ecotoxicology all measure some kind of damage to the DNA molecule (Shugart and Theodorakis, 1994; Shugart, 2000). These structural modifications to DNA

by chemicals include: strand breakage, dimerization of thymines, formation of DNA cross-links, DNA adducts and abasic sites. Genotoxicants can also interfere with DNA repair, replication and methylation leading to mutation and to epigenetic changes (Shugart, 2000). Some of these modifications are specific of the chemical that induced them. This is the case, for example, for the formation of adducts that covalently bond the genotoxicant and DNA, or for the dimerization of pyrimidine bases which typically follows the exposure to ultraviolet light in the range 290–320 nm. Other biomarkers, such as strand breaks, are not specific of a particular genotoxicant; the agent responsible for the genotoxic effect will have to be identified through different analytical techniques.

What follow is a list of the main biomarkers and the techniques that have been used in eco-genotoxicological studies of birds. A complete list of potential biomarkers would go beyond the scopes of this review, and the reader should consult pre-existing reviews from the toxicological and ecotoxicological literature (Bock, 2009; Chaudhry, 2008; Fossi, 1994; Hwang and Kim, 2007; Jha, 2004; Shugart, 2000).

### 2.1 Adduct formation

A DNA adduct is formed when a specific genotoxicant that has become bioavailable bonds covalently to the DNA (Shugart, 2000), which typically happens in proportion to the concentration of the genotoxicant (or of its metabolites), thus serving as a dosimeter for that particular genotoxicant. There are several techniques for the detection of DNA adducts, including high performance liquid chromatography (HPLC) and immunoassays (Qu et al., 1997). The most common protocol, however, is the so-called  $^{32}\text{P}$ -postlabeling (Shugart, 2000).  $^{32}\text{P}$ -postlabeling consists of a few steps, starting with hydrolysis of isolated DNA through an enzymatic treatment, with resulting formation of 3'-monophosphates that are  $^{32}\text{P}$ -labeled. Labeled adducts are then distinguished from normal nucleotides through thin-layer-chromatography. The adducts can then be detected after placing the chromatogram on a X-ray film, and their levels can be quantified through scintillation counting (Qu et al., 1997). In addition to its high specificity, this assay is also extremely sensitive, with the capability of detecting adducts at frequencies as low as one every  $10^{10}$  normal bases (Qu et al., 1997).

### 2.2 DNA strand breakage

DNA strand breakage is a relatively common category of DNA damage, which can naturally occur within a cell due to metabolic processes inside of it. Compared

to the measurement of DNA adducts, the quantification of DNA strand breakage is thus typically non-specific to the genotoxicant being investigated. However, modifications of some assays through the addition of enzymatic steps allow the detection of breaks of specific etiology, e.g. damage of oxidative origin (Collins, 2009). The general principle behind most assays that detect DNA strand breakage is that single-stranded DNA, after denaturation at high pH, detaches and extends from its supercoiling and its duplex structure in a way that is proportional to the number of its strand breaks (Rydberg 1975).

Also termed single-cell gel electrophoresis (SCGE) assay, the comet assay has rapidly become the most common technique for the detection of DNA strand breakage. The comet assay is simple, inexpensive and reliable, and can be applied to virtually any nucleated cell line (Cotelle and Ferard, 1999; Dhawan et al., 2009; Fairbairn et al., 1995; Tice et al., 2000). The significance of this assay in genetic toxicology has been the subject of other excellent reviews (Cotelle and Ferard, 1999; Frenzilli et al., 2009; Jha, 2008; Kleinjans and van Schooten, 2002). Therefore, only some details and benefits will be emphasized here.

One of the benefits of this assay is that the observations are conducted at the level of single cells, as is typical of cytogenetic assays. Other advantages include the fact that no prior knowledge of the karyotype and of cell turnover rate is required, and that cells do not need to be in any specific phase of the cell cycle, differently from other cytogenetic techniques (e.g. the micronucleus assay, the sister-chromatid exchange test).

There are two main versions of the comet assay, an alkaline version (Singh et al., 1988), which is the most frequently used, and a neutral version. The alkaline protocol can detect both double-strand breakages (DSBs) and single-strand breakages (SSBs) to the DNA molecule, including those associated with incomplete excision repair sites, as well as alkali-labile sites (ALS), DNA-DNA and DNA-protein cross-linking (Hartmann et al., 2003). The neutral version of the assay only allows for the detection of DSBs (Tice et al., 2000). The assay consists of a few steps. It starts with embedding cells in agarose at a concentration that varies, but whose main requirement is that cells are frequent enough that hundreds of them can be successfully measured, but not as frequent as to overlap with each other. The cells are then lysed in high alkali solution (pH > 13.0 in the alkaline version), and DNA is freed from the scaffolding is left free to migrate in an electrophoretic field. The dis-

tance migrated during electrophoresis is linearly related to the degree of damage to DNA (Fairbairn et al., 1995).

The specificity and sensitivity of the comet assay can also be improved through the addition of enzymatic steps that convert specific lesions to breaks: a treatment with Endonuclease III (EndoIII), a DNA repair enzyme, can be used to transform oxidized pyrimidines in SBs, while the enzyme formamidopyrimidine DNA glycosylase (FPG) can be used to detect 8-oxoguanines and other damaged purines (Azqueta et al., 2013).

Different metrics can be used for evaluating damage at the level of single cells, although there seems to be growing consensus around the use of the proportion of DNA in the tail of the 'comet', a more straightforward metric compared to composite measurements such as the 'tail moment' or the 'Olive tail moment' (Kumaravel et al., 2009; Tice et al., 2000).

Although certainly a versatile test, the comet assay has its own shortcomings. Results of the assay are highly dependent on the details of the protocol (Azqueta and Collins, 2013; Azqueta et al., 2011), particularly when it comes to voltage and duration of electrophoresis, but also for agarose concentration (Azqueta and Collins, 2013). Thus, a certain degree of optimization is required, especially for non-model species (Jha, 2008), as is the case for birds.

### 2.3 Chromosomal aberrations and other cytogenetic anomalies

There are many kinds of cytogenetic abnormalities, the main ones being chromosomal aberrations (CA), sister chromatid exchanges, micronuclei and aneuploidy (Tucker and Preston, 1996). They either result from the interference of genotoxicants with the process of segregation of chromosomes during cell division, or from misrepaired DSBs (Obe et al., 2002). Biomarkers of these categories of DNA damage, which typically fall in the category of 'biomarkers of exposure' linearly track exposure with remarkable accuracy. In birds, as in all eukaryotic cells, there are three main pathways for DSB repair: (i) non-homologous end-joining (NHEJ), a relatively inaccurate pathway that introduces substitutions, insertions and deletions at the break site; (ii) single strand-annealing, and (iii) homologous recombination repair, a process capable of accurately restoring the initial DNA sequence (Obe et al., 2002). While several techniques are available for the detection and characterization of cytogenetic abnormalities (Tucker and Preston, 1996), including fluorescence in situ hybridization (FISH) (Swiger and Tucker, 1996), perhaps the most frequently utilized in ecotoxicological studies is flow

cytometry (Dallas and Evans, 1990). This is a highly versatile, fast and relatively inexpensive technique for the detection of a wide range of damage categories (see below). It is then unsurprising that flow cytometry was the technique chosen by a few ecogenotoxicological studies that assessed exposure of bird species to a range of different contaminants, from heavy metals to pesticides to aromatic compounds (Custer et al., 2000; 2006; Wickliffe and Bickham, 1998).

#### 2.3.1 Flow cytometry

Flow cytometry is a non-specific assay that, in addition to chromosomal aberrations and sister-chromatid exchanges, can also quantify DNA strand breakage and adducts (Dallas and Evans, 1990). The premise at the basis of the assay is that a genotoxicant can unbalance the distribution of the genetic material during mitosis: its interference during mitosis will result in a higher coefficient of variation of cellular DNA content for cells that are in the  $G_0/G_1$  phase of the cellular cycle (Dallas and Evans, 1990). Flow cytometry can also be used for quantifying the RNA content of cells, and the population of aneuploid cells (Dallas and Evans, 1990). One other advantage of this protocol consists in the possibility of analyzing a large number of cells, as 10,000 cells can typically be measured in the span of a few seconds (Dallas and Evans, 1990).

#### 2.3.2 Micronuclei

Micronuclei (MN) are small bodies that originate outside the nucleus by chromosome breakage and centromere or spindle dysfunction during cell division. They are generated by both aneugens, which induce spindle damage, and clastogens, which cause chromosome to break (Fenech, 2000). This test has long been used as a biomarker of exposure, and is generally considered to be reliable and sensitive. Birds have been shown to have low levels of spontaneous MN (Zúñiga-González et al., 2000; 2001). Thus, high levels of micronuclei are likely due to exposure to a genotoxicant, and their detection is not confounded by other environmental factors and/or by inter-individual differences in age, sex, nutritional state or physiological condition.

## 3 Ecogenotoxicological Studies of Birds

As previously mentioned, the taxonomic diversity and the variety of contexts across studies support the validity of birds as monitors of genotoxic contamination. This is even more remarkable given the undeniable overall paucity of eco-genotoxicological studies of birds. We will now provide an overview of these studies, in

the hope to clarify the scope for the application of genotoxicological techniques to bird species. This overview intentionally omits those studies that have assessed genotoxic effects of chemicals strictly under laboratory conditions, without reference to wild populations of animals, such as the many studies on embryo toxicity in the chicken *Gallus gallus*. The studies are also listed in Table 1, where a brief outline of the different protocols, together with some summary considerations on the usefulness of each biomarker is provided.

In a series of studies that spanned several years, scientists measured DNA damage following the Doñana ecological disaster, a spill of acid waste from a pyrite ore mining site (Pain et al., 1998), in two species that occupy a high level within the food web, namely the white stork *Ciconia ciconia* and the black kite *Milvus migrans* (Pastor et al., 2001b; 2004; 2001a), both of which feed on a variety of vertebrate and invertebrate species. These studies showed that the young of both species had increased levels of DNA damage in their

lymphocytes when compared to birds from uncontaminated sites (Pastor et al., 2001b; 2004; 2001a). A similar approach was used to test for an increase in genetic damage with increasing levels of radioactive contamination in barn swallows *Hirundo rustica* from the Chernobyl region (Bonisoli Alquati et al., 2010). Ionizing radiation can exert genotoxic effects directly, by inducing DNA single strand breaks (SSBs) and double strand breaks (DSBs), or by increasing levels of reactive oxygen species (ROS), which in turn can damage macromolecules including nucleic acids if left unquenched by the antioxidant system (Barzilai and Yamamoto, 2004). The barn swallow was also at the center of a study that assessed the concentration of polychlorinated biphenyls (PCBs) in eggs and nestlings collected along the most polluted waterway of the Calcasieu Estuary in Louisiana (Custer et al., 2006). In this case, however, while the concentrations of PCBs were significantly higher in contaminated sites compared to control sites, DNA damage, as measured through flow cytometry of peripheral blood, was not elevated in contaminated sites

**Table 1** Methods used to estimate genotoxicity of environmental contaminants in birds species, together with the corresponding references

Biomarker	Protocol	Tissue	Principle and notes on applicability	References
Adduct formation	<sup>32</sup> P-postlabelling	Any cell line	Based on the binding of chemicals to the DNA molecule. The high specificity allows the chemical characterization of the genotoxicant; high sensitivity and broad applicability across species	1, 2
Strand breakage	Single cell gel electrophoresis (or Comet assay)	Virtually any cell line	Inexpensive, sensitive and reliable; it allows detection of different classes of DNA damage (including alkali-labile sites, excision-repair sites, single and double-strand breakages); non-specific of any genotoxicant, but enzymatic steps allow for the selective expression of certain categories of damage (e.g. damage of oxidative origin). Sensitivity to experimental conditions requires optimization of the test.	3–7
Cytogenetic abnormalities	Flow cytometry	Blood	Detection of an unbalance in the distribution of DNA among cells. A non-specific test that rapidly scans high numbers of cells, with very high sensitivity.	8–10
Cytogenetic abnormalities	Micronuclei (MN)	Blood	MN are small abnormal bodies that originate by chromosome breakage and centromere or spindle dysfunction during cell division. They allow the sensitive and reliable detection of both aneugens and clastogens. Low levels of spontaneous MN in birds allow ruling out the intervention of confounding environmental factors.	1, 8
Mutation	Mutation rate at neutral or coding loci	Somatic or germinal cell line	A suite of different techniques is available, with varying sensitivity depending on the degree of coverage of the genome. Downstream to the DNA-genotoxicant interaction, it signals a failure of the cellular repair system; potentially more linked to population-level dynamics if functional genes are selected.	11–14

1. Skarphedinsdottir H et al. (2010) *Mutat. Res.* 702: 24–31. 2. Schilderman PA et al. (1997) *Environ. Health Persp.* 105: 322–330. 3. Bonisoli Alquati A et al. (2010) *Comp. Biochem. Phys. C* 151: 271–277. 4. Pastor N et al. (2004) *Mutagenesis* 19: 61–65. 5. Pastor N et al. (2001) *Mutagenesis* 16: 219–223. 6. Siculo M et al. (2010) *Arch. Env. Contam. Toxicol.* 59: 484–491. 7. Maness T, Emslie S (2001) *Waterbirds* 24: 352–360. 8. Ilyinskikh NN et al. (1997) *Environ. Pollut.* 98: 119–122. 9. Wickliffe JK, Bickham JW (1998) *Bull. Environ. Contam. Toxicol.* 61: 239–246. 10. Custer TW et al. (2006) *Environ. Monit. Assess.* 121: 543–560. 11. Eeva T et al. (2006) *Mutat. Res.* 608: 8–15. 12. Ellegren H et al. (1997) *Nature* 389: 593–596. 13. Yauk CL, Quinn JS (1996) *PNAS* 93:12137–12141. 14. Yauk CL et al. (2000) *Mutat. Res.* 452: 211–218.

(Custer et al., 2006). Flow cytometry was also used for analyzing DNA damage following exposure to planar halogenated hydrocarbons and heavy metals in brown pelicans *Pelecanus occidentalis* living along the North Carolina coast (Wickliffe and Bickham, 1998).

A few studies have used pigeons *Columba livia* to investigate the potential genotoxicological effects of airborne contaminants, with the aim of evaluating whether this species can be used as an ecological indicator of the levels of traffic-related contaminants (Schilderman et al., 1997; Siculo et al., 2010). The pollutants that were tested included polycyclic aromatic hydrocarbons (PAH), ozone, CO, NO<sub>2</sub>, and heavy metal residues. Both studies concluded that pigeons are useful monitors of airborne contaminants, as higher levels of the contaminants caused an increase in oxidative damage to the DNA and higher levels of polycyclic aromatic hydrocarbons (PAH)-DNA adducts (Schilderman et al., 1997), while genetic damage was significantly higher in pigeons living outdoors compared to controls raised in clean, indoor conditions (Siculo et al., 2010). The formation of DNA-PAH adducts was also used to assess exposure to genotoxicants in herring gulls *Larus argentatus* from urban (= contaminated) and rural (= clean) sites in Iceland and Sweden (Skarphedinsdottir et al., 2010). Higher adducts in urban populations demonstrated exposure to genotoxicants, confirmed also by an increase in the frequency of micronucleated erythrocytes (Skarphedinsdottir et al., 2010). The study also demonstrated that the levels of DNA adducts in the liver of herring gulls were higher than in fish, thus supporting the role of birds as sensitive sentinels of genotoxic contamination (Skarphedinsdottir et al., 2010).

Micronucleated erythrocytes increased in frequency with exposure to radioactive contamination in an impressive sample of >15,000 pigeons from a large region contaminated by a fallout from the Siberian Chemical Plant, near the city of Tomsk, in Russia (Ilyinskikh et al., 1997). Individuals from sites located downwind from the plant had higher frequency of micronuclei compared to an uncontaminated site, with a clear gradient of increasing frequency at shorter distances from the plant (Ilyinskikh et al., 1997).

In addition to genetic damage, mutational events have also been used as biomarkers of exposure to various genotoxic chemicals in birds. This was the case of seagulls living in an heavily industrialized urban area, which were found to have increased heritable minisatellite mutation rate (Yauk et al., 2000; Yauk and Quinn, 1996). Similarly, barn swallows exposed to low-level

radioactive contamination following the Chernobyl accident had a microsatellite mutation rate two- to ten-fold higher compared to control populations across Europe (Ellegren et al., 1997). Low-level radioactivity was also found to increase nucleotide diversity in pied flycatchers *Ficedula hypoleuca* living close to a site of uranium and plutonium processing contaminated by radioactive fallout, in Russia (Eeva et al., 2006). Interestingly, in the same study great tit (*Parus major*) living close to a smelter site (Harjavalta, Finland) also showed an increase in nucleotide diversity compared to populations from control sites, while pied flycatchers from the same and another smelter site showed lower nucleotide diversity compared to clean sites (Eeva et al., 2006). In these two same species, the same authors had previously tested a number of genotoxicity biomarkers along a gradient of heavy metal contamination in what is probably the most integrated and thorough ecogenotoxicological study ever conducted in a bird species (Eeva et al., 2000). Eeva et al. (2000) analyzed the enzymatic activity of ethoxyresorufin O-deethylase (EROD), and delta-aminolevulinic acid dehydratase (ALA-d), two detoxifying enzymes, while also assessing the breeding performance of birds, used as an index of their ecological response. In flycatcher nestlings, but not in great tit nestlings, the authors discovered higher EROD activity near the pollution source, while breeding success was also declining along the pollution gradient for both species (Eeva et al., 2000).

#### 4 Integration between the Biomarkers and Life History

An important aspect in the choice of the biomarker(s) to be used in ecogenotoxicological studies is the relationship between the biomarker and individual fitness (Jha, 2008). In other words, the biological and ecological significance of a biomarker will depend on the likelihood that its increased expression translates into deleterious fitness consequences for the organism and its population (Anderson et al., 1994).

Biomarkers that signal a deterioration of a body function or a reduction in fertility should thus generally be preferred to more neutral markers. This argument naturally favors the choice of 'biomarkers of effect' over those that track 'exposure' (see above) when risk assessment is to be made. In this case, by definition, the physiological, cellular and genetic responses that are tracked by the 'biomarkers of effect' are one step closer to the organismal response (Shugart, 2000). A clear relationship between a change in the biomarker and a fit-

ness effect has only rarely – if ever – been shown in any organism, and birds are no exception. In fact, individual genetic biomarkers are not necessarily expected to translate in direct fitness effects, or to be directly linked to population/community dynamics (Forbes et al., 2006). Yet this limitation to the prognostic capacity of biomarkers should not be considered a reason for discarding their use in ecological risk assessment. Rather, it should be considered a reason for critically coordinating the study of biomarker with the use of other tools, including the direct assessment of population dynamics and the development of statistical and computational models (Allen and Moore, 2004; Forbes et al., 2006).

Whether due to the primary attack by the genotoxigenic or to misrepair by the cellular DNA repair machinery, DNA damage itself can have relevant implications for body functions, mating success, fertility and survival. Recent advancements in sexual selection theory are placing great emphasis on DNA damage and its relationship to fitness, positing that a male's secondary sexual traits (SSTs) reveal his genetic resistance to oxidative stress and the integrity of DNA in his germ line (Blount et al., 2001; Schantz et al., 1999; Velando et al., 2008). Female preference for male SSTs would have evolved to allow females to avoid infertility, as well as genetic abnormalities, neoplasia and congenital malformations in their offspring (Velando et al., 2008; Wyrobek et al., 2006).

Under this hypothesis, variation in the resistance of DNA to oxidative damage should be correlated with differences in the expression of sexual signals. Interestingly, a study of the common yellowthroat *Geothlypis trichas* using the comet assay to measure DNA resistance to oxidative stress in erythrocytes recently confirmed a prediction stemming from this hypothesis by showing that males with showier plumage had higher resistance to genetic damage (Freeman-Gallant et al., 2011). Additional support also came from a study that linked plumage brightness to sperm quality: less colorful males had relatively higher levels of sperm peroxidation, but their sperm motility was more likely to increase if their diet was supplemented with antioxidants (Helfenstein et al., 2010). What remains to be tested is whether an increase in somatic and/or germline DNA damage would be mirrored by males' showiness and female preference for males, a task that can be answered only by using biomarkers of DNA damage developed in the context of environmental monitoring. Biomarkers of genotoxicity can thus provide tools for basic research on the mechanisms of sexual selection, and the genetic and

physiological correlates of male showiness. DNA damage detected by the comet assay has been shown to be higher in snake species with higher pace of life (Bronikowski, 2008), thus supporting the use of this test for investigating the mechanistic basis of life-history evolution.

Differently from toxicological studies in humans, it is the fate of natural populations, rather than individual health, that is at the main focus of ecotoxicology (Anderson et al., 1994; Guttman 1994; Shugart and Theodorakis, 1994). Thus, as much as possible, the analysis of a biomarker of genotoxicity should be associated with its population level implications, whether inferred or measured. Alternatively, genotoxicity can be assessed by using changes in the genetic composition of the affected population (Depledge, 1994; Shugart and Theodorakis, 1994). A few studies have attempted or demonstrated that such translation can take place (Atienzar et al., 2001), and even influence community composition (Crowe et al., 2004).

At the community level, species variation in susceptibility to genotoxigens can then allow the identification of reliable biomonitors of environmental contamination and impact our understanding of variation across species in ecological traits and life history adaptations. Genotoxic contamination can be assimilated to a stressor or an episode of ecological disturbance that creates quasi-experimental conditions by exacerbating the trade-offs between competing physiological and life history functions, thus providing a glimpse at the underlying physiological and cellular mechanisms.

An example of these 'natural' laboratory is the landscape of radioactive contamination created by the Chernobyl accident in 1986 (Møller and Mousseau, 2006). A study of barn swallows in the Chernobyl region capitalized on increased mutation rate in this species to investigate the relationship between mutational load and sexual selection (Møller and Mousseau, 2003). Similarly, censuses of species abundance in Chernobyl assessed the susceptibility of bird species to radioactive contamination, and demonstrated that the ecological fate of different bird species in the wake of the nuclear disaster could be predicted by their ecological and life history traits, including migratory and dispersal behavior, the intensity of sexual selection and maternal pre natal care of the offspring (Møller and Mousseau, 2007), as well as the requirement for glutathione (GSH) (Galván et al., 2011).

In turn, this wealth of data on interspecific variation in susceptibility to genotoxigens could provide impor-



tant information for investigating the mechanistic bases of life history evolution. It has been increasingly recognized that oxidative stress, i.e. the imbalance between the production of reactive oxygen species (ROS) and their neutralization by the antioxidant system (Costantini, 2008), is the currency underlying life history trade-offs and aging (Buttemer et al., 2010; Costantini 2008; Dowling and Simmons, 2009; Monaghan et al., 2009). Comparative evidence indicates that typical longevity of a species positively correlates with its cellular resistance to oxidative damage, through gene transcription and translation (Holmes et al., 2001; Ogburn et al., 2001). As the main deleterious action of ROS is to damage macromolecules including DNA, variation across species in resilience to oxidative damage and in DNA repair efficiency can also help understanding their variation in life history (Dowling and Simmons, 2009). Once again, evidence of the link between antioxidant physiology and life history variation comes from avian studies in Chernobyl. All the ecological traits that were found to determine the severity of species decline in the region were linked to the requirement for antioxidants (Galván et al., 2011; Møller and Mousseau, 2007). Species with relatively high needs for antioxidants were likely to suffer from the additional ROS generated by ionizing radiation. If that is true, then DNA damage induced by higher levels of ROS that cannot be quenched by the antioxidant system could predict the ecological fate of a species following exposure to nuclear fallout. We are currently testing this hypothesis by analyzing DNA damage data – as measured by the comet assay – in several hundreds birds from more than thirty different bird species. This database would be the largest set of data on the interspecific variation in susceptibility to a genotoxicant in any taxon, and we expect that it will not only inform future choices of the indicator species in studies monitoring the ecological effects of radioactive contamination, but also provide valuable insight on the link between physiology and life history.

In this context, a DNA modification that has the potential of serving as a biomarker of genotoxicity while also having demonstrated ties to life history variation is the loss of telomeres, the repetitive DNA sequences at the end of the linear chromosomes of eukaryotes (Monaghan, 2010; Monaghan and Haussmann, 2006). Telomere length has been implicated in a number of life history traits of birds, most notably individual rate of senescence (Haussmann et al., 2003) and survival prospects (Salomons et al., 2009; Vleck et al., 2011), which telomere predict even more accurately than chronologi-

cal age of the bird itself (Bize et al., 2009). Such predictions can also be formulated on the basis of the length of telomeres at an early age (Hall et al., 2004; Heidinger et al., 2012). Chemicals that impact telomeres can thus have consequences for cellular and organismal senescence. Some contaminants have been shown to accelerate the rate of telomere shortening (or ‘telomere attrition’), through induction of oxidative stress (Liu et al., 2003). This idea, however, remain largely untested in any bird species, and even more generally in any wild population of animals.

## 5 Future Developments in Biomarkers of DNA Damage

Given the paucity of genetic ecotoxicological studies of birds, particularly compared to marine and freshwater taxa, a lot remain to be studied in birds. This is especially true for the general lack of information concerning intra- and interspecific variation in susceptibility and in response to genotoxicants. Future developments include the assessment of the genotoxic effect of contaminants on tissues other than blood, which has been the choice of most studies. The liver, where much of the detoxification takes place, and the brain, which could be mediating higher-level effects on behavior, are obvious sensitive targets for monitoring exposure to genotoxicants and its associated risks, as are the reproductive organs, due to potential transgenerational consequences of impacting their tissues. An obvious complication is that sampling of such organs would not be as non-destructive as blood collection (but see Sheldon et al., 2008).

A large scope also exists for the application of high-throughput techniques, particularly those belonging to the so-called ‘-omics revolution’. Genomic resources for bird studies have been recently expanding (Backström et al., 2008; Santure et al., 2011; van Bers et al., 2012), providing resources for genome-wide scans that can allow the comparison of single nucleotide polymorphism (SNP) level and scan for the expression of a multitude of genes (Waters and Fostel, 2004). The revolution promised by ‘toxicogenomics’, although not without challenges (Fielden and Zacharewski, 2001; Orphanides, 2003), will be at least twofold: on one side these high-throughput screening tools will allow much greater power to detect mutational events and sublethal effects of the genotoxicant; on the other side, they will grant unprecedented, system-wide insight into the mechanisms of action of the genotoxicant (Waters and Fostel, 2004). Importantly, some of the few genomic

resources that are currently available for bird species have demonstrated potential for cross-species microarray (CSM) analyses, as shown in a study where an array designed for the zebra finch *Taeniopygia guttata* successfully hybridized with genomic DNA from the common whitethroat *Sylvia communis* (Naurin et al. 2008). In spite of the progressive reduction in costs, genomic techniques are still considerably expensive, with sample processing costs in the order of hundreds USD (i.e. at least an order of magnitude higher than with traditional analysis of polymorphism with neutral markers or by restricting the screening to a few, meaningful genes). It is perhaps a bit early to coin the term 'ecotoxicogenomics'.

Another important development of toxicology that has a large potential to influence ecogenotoxicological studies is the analysis of DNA methylation patterns (Vandegheuchte and Janssen, 2011). The exposure to persistent organic pollutants, endocrine disruptors and other genotoxicants can leave epigenetic marks, which become mitotically or meiotically heritable (Vandegheuchte and Janssen, 2011). A promising aspect of the use of epigenetics in ecotoxicological studies is, in fact, its potential to detect transgenerational effects of exposure to a genotoxicant. Evidence from laboratory studies point at the existence of large variation across species in the levels and patterns of methylation (Vandegheuchte and Janssen, 2011), thus suggesting the scope for the identification of sentinel species.

While more sensitive and specific biomarkers are being devised, research efforts should also be devoted to understand the mechanistic basis of the genotoxic effects of chemicals, as well as the behavioral and ecological correlates of exposure and susceptibility. This mechanistic understanding cannot forego the contribution of controlled studies, which can control a suite of confounding factors including genetic and developmental variation, age and life stage, nutritional, physiological and immune status (Forbes et al., 2006). It should be kept in mind, however, that realistic ecological conditions are likely to exacerbate the stress originating from exposure to a given genotoxicant. Thus, the extrapolation of toxicological benchmarks for wildlife from corresponding studies conducted under relatively benign lab conditions or in a controlled environment can lead to an underestimation of the ecological risk implied by the dispersion of a chemical. This was the conclusion of a recent review of studies of low dose radiation exposure that compared radiosensitivity data from field studies and controlled exposure experiments (Garnier-

Laplace et al., 2012). Although genotoxicity endpoints were not included in the review, under the assumption that they would not translate into population-level effects, the review demonstrated that hazardous dose rates estimated under field conditions were eight times lower than under controlled conditions (Garnier-Laplace et al., 2012). Allowing radionuclide levels that prove safe under controlled conditions may not be a good idea for 'real world' organisms.

In addition, addressing the mechanistic basis of genotoxicity and the response of a genome to toxic substances will require a better understanding of the timing and the shape of the dose-response curve. The 'paradigm' of a linear relationship between the dose of the genotoxicant and the expression of the biomarker has been increasingly challenged by the observation that organismal sensitivity and response can be best represented by non-monotonic, 'J-' or 'U-shaped' curves (Calabrese, 2008). There is increasing evidence that a wide variety of stressors elicit a biphasic response from organisms, with low doses exerting priming and/or stimulating effects, while high doses inhibit an organism's response – or, in other words, become toxic (Calabrese, 2008; Mattson and Calabrese, 2009). This phenomenon is termed 'hormesis' and a wealth of ecological and evolutionary studies has recently documented hormetic-like responses to stressors under natural conditions, which perhaps evolved as a mechanism enabling phenotypic plasticity (Costantini et al., 2010). Thus, the choice of the biomarkers of genotoxicity and of a reliable bioindicator species require that the time scale of exposure be considered, and that the possibility of a beneficial effect of exposure is also considered (but see Calabrese and Baldwin 2002 on the distinction between a stimulatory response and beneficial effects.)

## 6 Conclusions

In this review, I argue that bird species provide excellent indicators of the ecological risk associated with genotoxicants. The arguments that make bird species excellent biomonitors in genotoxicological studies largely overlap with traditional arguments that have been put forward to justify the use of the birds for environmental monitoring (Burger and Gochfeld, 2004; Furness and Camphuysen, 1997; Furness and Camphuysen, 1997). These reasons are fundamentally practical arguments that emphasize the ecological diversity, approachability and conspicuousness of birds, as well as the possibility of capitalizing on previously collected specimens and information.

Although the number of eco-genotoxicological studies involving bird species is still limited, the techniques employed span a wide range of technologies and protocols, thus proving their general applicability to birds. We suggest that the wealth of information concerning the life history and the ecological traits of bird species can facilitate the identification of sensitive and reliable indicators of environmental contamination and ecological perturbation. Conversely, the information on intra- and interspecific variation in susceptibility to genotoxins in bird species will greatly benefit our understanding of the mechanistic bases of variation in life history.

The use of birds as monitors in ecogenotoxicological studies is not without challenges, and a lot remains to be done, particularly with recent technological advances for high-throughput screening and analysis. The synanthropic ecology of many bird species, which adapted to exploit food resources and shelter provided by human settlements, exposes them to the same contaminants that threaten human health, thus making them potentially good sentinels, provided that their exposure and/or sensitivity are higher than for humans. At present, however, the potential to extrapolate genotoxic risks from bird species to humans remains largely theoretical. Interestingly, when similar effects are disclosed for humans and wildlife, the findings in wildlife can support the existence of a direct link between the contaminant and the endpoint measured, thus excluding the intervention of confounding socio-economic factors. A similar argument has been put forward for exposure to radioactive contamination following the Chernobyl accident, where many of the effects on human health have been blamed on substance use and psychosocial distress following relocation (Mousseau and Møller, 2013). However, given the large variation in the response of biomarkers to contamination even among mammals (Baan et al. 1994), we should exercise caution in predicting an outcome for humans on the basis of a demonstrated ecological risk. Nonetheless, if a bioindicator is to provide an 'early warning' of a potential effect to human health, then the risk of a false alarm (i.e. a 'false positive', or a Type I error, in statistical terms) should be considered more acceptable than the risk of not detecting a threat (i.e. a 'false negative', or a Type II error).

Differently from when canaries were used as an early warning in coalmines, both humans and birds can benefit from the use of birds in modern eco-genotoxicological studies.

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