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RESEARCH ARTICLE

Linkages between the life-history evolution of tropical and temperate birds and the resistance of cultured skin fibroblasts to oxidative and non-oxidative chemical injury

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SUMMARY

A fundamental challenge facing physiological ecologists is to understand how variation in life history at the whole-organism level might be linked to cellular function. Thus, because tropical birds have higher annual survival and lower rates of metabolism, we hypothesized that cells from tropical species would have greater cellular resistance to chemical injury than cells from temperate species. We cultured dermal fibroblasts from 26 tropical and 26 temperate species of birds and examined cellular resistance to cadmium, H_2O_2, paraquat, thapsigargin, tunicamycium, methane methylsulfonate (MMS) and UV light. Using ANCOVA, we found that the values for the dose that killed 50% of cells (LD_{50}) from tropical birds were significantly higher for H_2O_2 and MMS. When we tested for significance using a generalized least squares approach accounting for phylogenetic relationships among species to model LD_{50}, we found that cells from tropical birds had greater tolerance for Cd, H_2O_2, paraquat, tunicamycin and MMS than cells from temperate birds. In contrast, tropical birds showed either lower or no difference in tolerance to thapsigargin and UV light in comparison with temperate birds. These findings are consistent with the idea that natural selection has uniquely fashioned cells of long-lived tropical bird species to be more resistant to forms of oxidative and non-oxidative stress than cells from shorter-lived temperate species.

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INTRODUCTION

Life-history theory posits that key events during an organism's life, such as the rate of juvenile development, age of first reproduction, number of offspring produced and the rate of senescence (Mitteldorf and Pepper, 2009; Longo et al., 2005), are shaped by natural selection to produce the largest possible number of surviving offspring (Stearns, 1992). Variation in life-history events is thought to reflect the differential allocation of resources, time and/or energy to competing life functions, largely growth, body maintenance and reproduction (Charnov, 1993; Ghalambor and Martin, 2001). Life-history variables are constrained within limited ecological space, lying along a 'slow–fast' life-history axis: animals that invest large quantities of resources in reproduction early in life generally die young, whereas animals that invest more resources in bodily maintenance tend to have fewer offspring and live longer (Saether, 1988; Promislow and Harvey, 1990; Ricklefs, 2000).

With low annual reproductive output and high annual survival rate, tropical birds lie at the slow end of the life-history continuum (Francis et al., 1998; Ricklefs, 1997; Brawn et al., 1998), whereas temperate birds tend to cluster more at the fast end of the spectrum, with large clutch sizes and high rates of mortality. For example, tropical house wrens (*Troglodytes musculus*) invest fewer resources in reproduction, and have lower basal and field metabolic rates and higher survival rates than temperate house wrens (*Troglodytes aedon*), consistent with the idea that tropical birds have a slow pace of life (Tieleman et al., 2006). Likewise, the yellow warbler (*Dendroica petechia*), whose range encompasses Alaska to Central America, one of the largest breeding distributions of any New World passerine, shows considerable variation in life history along its latitudinal gradient. Further, tropical mangrove warblers (*Dendroica erythracoides*), exhibited significantly smaller clutch sizes, longer incubation and nestling periods, and higher annual adult survival rates than temperate yellow warblers (Salgado-Ortiz et al., 2008).

Although it is thought that physiological processes underlie many life-history trade-offs (Ricklefs and Wikelski, 2002), the precise linkages between an organism's life history and the function of its organs, tissues and cells remain obscure (Stearns, 1992; Speakman, 2005; Williams et al., 2010). Evidence of a linkage between slow pace of life and low rate of metabolism in tropical birds came to light when it was shown that they had a significantly lower whole-animal basal metabolic rate (Wiersma et al., 2007a) and peak metabolic rate as measured by cold-exposure or by exercise (Wiersma et al., 2007b). Later it was discovered that a contributing factor to the reduced rate of metabolism in tropical birds was their smaller metabolically active organs, such as the heart, liver, kidneys and pectoral muscles, compared with similar-sized temperate species (Wiersma et al., 2012). These findings offer evidence of a connection between the life history of tropical birds and their physiology, at least at the organismal and organ levels. How the actual cells of tropical bird species might differ from temperate species as a result of their life history remains unknown.

A fundamental challenge facing physiological ecologists is an understanding of how variation in life history at the whole-organism level might be linked to cellular function (Williams et al., 2010). As a result of normal oxidative phosphorylation in the mitochondria, cells produce free-radicals such as $O_2^{\bullet}$, and other reactive molecules such as H_2O_2 (Harman, 2001), which can attack DNA, proteins and lipids, causing impairment of function and ultimately cell death if the damage cannot be repaired. Over time, damage from these reactive oxygen species (ROS) is thought to be an important aspect of the aging process, encapsulated in the free-radical theory of aging (Huang and Manton, 2004; Fukagawa, 1999; Finkel and Holbrook, 2000; Golden et al., 2002; Dufour and Larsson, 2004). Some level of oxidants, particularly H_2O_2, is thought to be essential for cell survival because of their role in gene regulation, cell signaling and apoptosis (Sohal and Orr, 2012). However, cells also have a number of mechanisms to combat oxidative stress and scavenge free radicals, including antioxidants such as glutathione peroxidase, catalase and super oxide dismutase (Finkel and Holbrook, 2000; Sohal and Orr, 2012). It is thought that the production of potentially harmful ROS and the concentration of antioxidants exist in a balance, which is important in the aging process (Sohal and Orr, 2012).

A corollary of the free-radical theory of aging is the idea that the cells of long-lived animals should be constructed in such a way as to resist ROS better than cells from short-lived animals. To test this idea, Kapahi et al. (Kapahi et al., 1999) examined the resistance of primary fibroblasts of eight species of mammals, ranging in body mass from 0.1 to 450 kg, to chemicals that imposed oxidative and non-oxidative stress. Even with such a small sample size, they found that cells from large, long-lived mammals resisted chemical stress agents better than cells from small short-lived mammals, a finding later supported by results from skin-derived fibroblasts of both rodents (Harper et al., 2007) and birds (Harper et al., 2011). Furthermore, cells from renal epithelium isolated from long-lived birds have also been found to have higher resistance to oxidative stressors (Ogburn et al., 1998). Interpretation of the above studies on mammals is often confounded by the fact that large animals tend to live longer than small ones, and that large animals also have cells with a low mass-specific rate of metabolism, thereby influencing exposure to stress agents. In the present study, we overcome these two problems and address whether differences in life-history trade-offs influence cellular resistance to chemical insult, such as oxidative stress, in tropical and temperate birds of similar body sizes. Furthermore, we explicitly account for the phylogenetic relationships among bird taxa in our statistical models.

We hypothesized that primary cell lines derived from tropical species of birds would be more resistant to chemical stress agents than cell lines derived from their temperate counterparts. Using conventional and phylogenetically adjusted statistical analyses, we found that cells from 26 species of tropical birds were generally more resistant to multiple forms of oxidative and non-oxidative stress compared with 26 species of temperate birds. These results suggest that tropical birds with their slow pace of life have evolved cells with a chemical make-up that resists forms of chemical injury more effectively that cells from temperate birds.

MATERIALS AND METHODS

Collection of birds

Tropical birds were collected by mist net in the lowland forest around Gamboa, Panama (9.12°N, 79.72°W), and temperate birds were collected by mist net, or salvaged as dead by catch, in or around the state of Ohio, USA. All birds that we collected were adults of unknown age. All procedures were approved by the Institutional Animal Care and Use Committee of The Ohio State University (protocol IACUC2004A0093) and capture of birds in Panama was accomplished under permit from the Panamanian Autoridad Nacional del Ambiente (no. SEX/A-22-12) and Autoridad del Canal de Panamá. Tissues were exported from Panama under USDA permit 118465.

Establishment of cell lines

We evaluated primary cell lines from 26 temperate and 26 tropical bird species for their resistance to a variety of chemicals that induce cell injury or metabolic stress: Cd, H_2O_2, paraquat, thapsigargin, tunicamycium, methane methylsulfonate (MMS) and UV light. For all species, primary cell cultures were established from the skin of free-living adult birds. Immediately after birds were killed, their feathers were plucked and the exposed skin was washed with antimicrobial soap. We excised a $5\times5\,mm^2$ piece of skin and placed it into cold complete bird cell culture medium [Dulbecco's modified Eagle medium (DMEM), high-glucose variant ($4.5\,mg\,ml^{-1}$), with sodium pyruvate ($110\,mg\,l^{-1}$), supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum and antibiotics ($100\,U\,ml^{-1}$ pen/strep), containing $10\,mmol\,l^{-1}$ HEPES].

We established primary fibroblast cell cultures after the skin was exposed to 1.5% Collagenase B solution overnight (Harper et al., 2007). Cells were grown in culture flasks at 37°C in an atmosphere of 3% O_2 (Harper et al., 2011). When cells reached 90% confluence, they were trypsinized (0.25%) and passaged. Seventy-five percent of the medium was replaced with fresh complete medium after day 3 in all flasks, and we split cells into new $75\,cm^2$ flasks at day 7–10. After cells grew for another 7 to 10 days, they were harvested and cryopreserved at 10^6 cells ml^{-1} in DMEM supplemented with 40% fetal bovine serum and DMSO at a final concentration of 10%. We stored cells in liquid N_2 for up to 12 months prior to assessment of their resistance to stress agents. All stress tests were conducted using cells at passage 4.

Assessment of fibroblast resistance to lethal stress

We tested cellular resistance to multiple agents using one to four cell lines for a given species. We seeded 3×10^4 cells in 100 µl of complete media into each well of a 96-well microtiter plate, allowing cells to attach for 24 h, and then exposed cells to DMEM lacking serum and sodium pyruvate but containing 2% bovine serum albumin for an additional 24 h. This step was added because the presence of fetal calf serum alters resistance to chemical insult from 3 to 20-fold (Harper et al., 2007). Thereafter, we exposed cells to graded doses of Cd, H_2O_2, paraquat, MMS, tunicamycin or thapsigargin for 6 h, or to graded doses of UV radiation. Cells were then washed with phosphate-buffered saline, and incubated in serum-free medium for an additional 18 h. Cell survival was evaluated using conversion of the extracellular tetrazolium dye WST-1 to its colored formazan product; inspection of the plates revealed a large number of dead cells at the higher concentrations of stress agents. Reference cells, incubated without any stress agent and representing 100% viability, were included in each day's set of assays and for each given cell line. All tests were carried out at 37°C in a humidified incubator with 5% CO_2 in 3% O_2.

We evaluated cellular resistance to a variety of chemicals that induce cell injury or metabolic stress. H_2O_2 causes OH^- radical formation in the presence of metal ions (Stohs and Bagchi, 1995), paraquat is a herbicide that induces $O_2^{\bullet}$ formation (Bus et al., 1976), thapsigargin inhibits Ca^{2+} pumping in the endoplasmic reticulum (Thastrup, 1990), tunicamycin is an antibiotic that interferes with

protein processing in the endoplasmic reticulum (Elbein, 1987) and MMS is a DNA alkylating agent (Harper et al., 2007). The heavy metal Cd indirectly generates the superoxide radical and hydroxyl radical, although the mechanism remains unclear (Galán et al., 2001). Some experiments suggest that Cd generates in cells hydrogen peroxide which may be a significant source of radicals *via* Fenton chemistry (Valko et al., 2005), and UV light provokes DNA strands to break causing misreading and mis-replication of genes (Griffiths et al., 1998).

Calculation of LD_{50} values

The resistance of each cell line to chemical stressors was calculated for duplicate wells run in parallel on the sample plate, with one plate per stressor. The concentration needed to obtain 50% survival of the cells, the LD_{50}, was calculated using the FORECAST function in Excel. Each of our statistical tests used mean LD_{50} values for a given measure as a representative for each species, even in cases when multiple cell lines were independently assessed.

Statistics

We used both conventional and phylogenetically controlled statistical analyses. First, to account for body mass effects on mean LD_{50}, per species and environment, all LD_{50} data were analyzed using an analysis of covariance (ANCOVA) with mean LD_{50} as the dependent variable and body mass as the covariate. Second, to determine whether tropical birds had greater cellular resistance to chemical insults relative to cells from temperate birds, we modeled mean LD_{50} per species for each stressor treatment as a function of environment (tropical or temperate) using generalized least squares (gls function in the R package nlme), including body mass as a covariate.

To account for the evolutionary relationships among species, we compared our basic model against four models in which the structure of the error term incorporated the phylogeny of the species by adjusting the variance–covariance matrix, thus taking into account the non-independence of data points as a result of evolutionary history (Paradis, 2006; Rohlf, 2001). Covariances can be manipulated into different models of trait evolution, each of which incorporates varying degrees of phylogenetic signal. Many models assume trait evolution that follows Brownian motion, where differences in traits between species are proportional to time since divergence (supplementary material Table S1) (Felsenstein, 1985). For our analyses, we also used Pagel's, Martins' and Grafen's models. Pagel's model modifies covariances between species by multiplying by a constant, λ; when traits are phylogenetically uncorrelated, $\lambda=0$, and when there is strong phylogenetic signal within the data, $\lambda=1$. For the latter, evolution is assumed to follow Brownian motion. Martin's model incorporates stabilizing selection, where trait covariances decrease exponentially with increasing time since divergence and where the strength of the directional selective force is controlled by the parameter α (Martins and Hansen, 1997). This model allows trait evolution to vary between non-directional Brownian motion ($\alpha=0$) and strong directional selection ($\alpha=1$). Grafen's model incorporates a calculation of branch lengths based on number of descendants (Grafen, 1989). The tree is scaled so that the root has a depth of 1, and branch lengths are raised to the power ρ. When $\rho=1$, a strong phylogenetic signal is implied.

For all five models of evolution (one model assuming no evolutionary relationships and four others assuming a variety of hypotheses), we tested a suite of five nested candidate models using an information theoretic approach (Burnham and Anderson, 2002). Rather than using *P*-values to assign significance to each parameter in the model, this approach uses likelihood methods to determine the best model, given the data, from a suite of candidate models. Our five candidate models included a full interaction model (environment × body mass, assuming a different relationship between LD_{50} and body mass for tropical and temperate birds), an additive model (environment + body mass, assuming different intercepts but identical slopes for tropical and temperate birds), environment as a single predictor (thus assuming only different mean LD_{50} values between tropical and temperate birds), body mass as a single predictor (assuming no difference between tropical and temperate birds) and the simplest null model assuming no relationship of LD_{50} to body mass or environment. We used the corrected Akaike's information criterion (AIC_c; a modification of AIC for small sample sizes) to differentiate among the five models, the model with the lowest AIC_c value being the best model that fits the data. The AIC_c values also allow us to compare across models of evolutionary hypotheses and determine whether adding a phylogenetically modified variance–covariance matrix to the basic model improves our understanding of the data.

All analyses were performed on untransformed LD_{50} values in R version 2.12 (R Development Core Team, 2011) using the package ape v. 2.6 (Paradis et al., 2004) to calculate phylogenetic correlation structures, the package nlme v. 3.1 (Pinheiro et al., 2012) for generalized least squares analyses and the package AICcmodavg v.1.24 (Mazerolle, 2011) for calculating AIC_c.

Phylogenies

For all species in our data set, we used a species-level tree (supplementary material Fig. S1). We constructed tree branch lengths from Sibley and Ahlquist (Sibley and Ahlquist, 1990) and modified the tree accordingly to recent literature (Johnson and Sorenson, 1999; Klicka et al., 2000; Yuri and Mindell, 2002; Boyd, 2011). Trees were manipulated using Mesquite (Maddison and Maddison, 2010).

RESULTS

Body mass for our sample of 52 species of tropical and temperate birds ranged from 2.9 to 613.0 g, a 200-fold range (Table 1). There was no relationship between LD_{50} values and body mass in all but one chemical stressor (UV; see Table 2, Fig. 1). Body mass had little effect on the majority of our results.

LD_{50} values showed wide variation, ranging from 14.7 to 464.8 $\mu mol\,l^{-1}$ for cadmium-treated cells, 13 to 500 $\mu mol\,l^{-1}$ for peroxide, 57 to 5000 $J\,m^{-2}$ for UV light, 1.2 to 188.5 $mmol\,l^{-1}$ for paraquat, 0.46 to 5.90 $mmol\,l^{-1}$ for MMS, 5.83 to 63.00 $\mu mol\,l^{-1}$ for thapsigargin and 18 to 385 $\mu g\,ml^{-1}$ for tunicamycin.

We found a significant effect of environment on mean LD_{50} values, with tropical birds having significantly higher values for a number of chemical stressors, although these results varied depending on the type of statistical analyses used. An ANCOVA, which takes into account body mass, revealed that LD_{50} for H_2O_2 ($P=0.013$) and MMS ($P=0.01$) were significantly higher in tropical birds.

Using generalized least squares, we selected the best model from a suite of five nested candidate models, ranging from a full model consisting of an interaction between environment (tropical *versus* temperate) and body mass, to the null model with no explanatory variables (supplementary material Table S1). Without accounting for phylogeny, the best model for six of the seven stressors (Cd, H_2O_2, paraquat, MMS, thapsigargin and tunicamycin) included only environment as a single predictor variable. In all of these six cases, tropical birds had higher LD_{50} estimates than temperate birds

Table 1. Common names, species names and body masses for each species of temperate and tropical birds in this study

Environment	Common name	Species	Body mass (g)
Temperate	Ruby-throated hummingbird	*Archilochus colubris*	2.9
	House wren	*Troglodytes aedon*	9.7
	Goldfinch	*Astragalinus tristis*	10.9
	Chipping sparrow	*Spizella passerina*	11.3
	Yellow warbler	*Dendroica petechia*	11.4
	Red-eyed vireo	*Vireo olivaceus*	13.6
	Barnswallow	*Hirundo rustica*	15.9
	Tree swallow	*Tachycineta bicolor*	17.8
	House finch	*Carpodacus mexicanus*	18.7
	Song sparrow	*Melospiza melodia*	19.7
	Nuthatch	*Sitta carolinensis*	20.5
	Downy woodpecker	*Picoides pubescens*	24.6
	House sparrow	*Passer domesticus*	26.1
	Horned lark	*Eremophila alpestris*	29.8
	Spotted sandpiper	*Actitis macularius*	30.6
	Grey catbird	*Dumetella cardinensis*	33.9
	Cowbird	*Molothrus ater*	42.9
	American robin	*Turdus migratorius*	70.7
	Killdeer	*Charadrius vociferus*	72.6
	European starling	*Sturnus vulgaris*	84.2
	Common grackle	*Quiscalus quiscula*	105
	Mourning dove	*Zenaida macroura*	105.9
	Green-winged teal	*Anas crecca*	343.8
	Wood duck	*Aix sponsa*	452.8
	Shoveler	*Anas clypeata*	613
Tropical	Rufous-tailed hummingbird	*Amizilia tzacatl*	2.9
	White-necked jacobin	*Florisuga mellivora*	7
	Variable seed eater	*Sporophila corvina*	10.9
	Ochre-bellied flycather	*Mionectes oleagineus*	11
	Mangrove swallow	*Tachycineta albilinea*	11.7
	Plain xenops	*Xenops minutus*	12
	Golden collared manakin	*Manacus vitellinus*	12.4
	House wren	*Troglodytes musculus*	13
	Spotted antbird	*Hylophylax naevioides*	18
	Social flycatcher	*Myiozetetes similis*	21.6
	Crimson-backed tanager	*Ramphocelus dimidiatus*	24.6
	Barred antshrike	*Thamnophilus doliatus*	25
	Panamanian flycatcher	*Myiarchus panamensis*	26
	Palm tanager	*Tangara palmarum*	32.2
	Blue-gray tanager	*Tangara episcopus*	33.2
	Ruddy ground dove	*Columbina talpacoti*	39.9
	Bright-rumped attilla	*Attila spadiceus*	40
	Red-crowned woodpecker	*Melanerpes rubricapillus*	43.5
	Buff-throated saltator	*Saltator maximus*	46
	Common pauraque	*Nyctidromus albicollis*	46.9
	Black-faced antthrush	*Formicarius analis*	59
	Buff-throated woodcreeper	*Xiphorhynchus guttatus*	64
	Clay-colored robin	*Turdus grayi*	70
	Northern jacana	*Jacana spinosa*	94
	White-tipped dove	*Leptotila verreauxi*	129
	Southern lapwing	*Vanellus chilensis*	283

(Table 2, Fig. 1). When exposed to MMS, the null model was within two AIC_c units of the environment-only model, suggesting that environment played a weaker role than for the other stressors. When exposed to UV, the best model was the full interaction model, but here tropical birds had slightly lower LD_{50} estimates than temperate birds.

When we accounted for the phylogenetic relationships among species by modifying the variance–covariance matrix in four different ways, there was little qualitative difference to the results from the basic models that assumed complete independence among species, although specific coefficient estimates differed in most cases (Table 2, Fig. 1). For models with variance–covariance matrices modified according to Pagel's, Martins' and Grafen's evolutionary hypotheses, the results were qualitatively identical in all but two cases. Tropical birds had higher LD_{50} values than temperate birds in response to Cd, H_2O_2, paraquat, MMS, thapsigargin and tunicamycin. When exposed to UV, for all phylogenetic models, the best model was the null model. Models assuming a Brownian motion model of evolution were not considered because they performed very poorly, usually with AIC_c values over 20 units greater than all other candidate models, indicating a far worse fit than models with no evolutionary hypothesis. Thus, statistical models accounting for the evolutionary relationships among bird species concurred with models assuming no evolutionary relationships: cells of tropical birds had higher tolerance to chemical stressors than cells of temperate birds.

Table 2. Results of phylogenetic generalized least squares models describing mean LD_{50} per chemical stressor treatment as a function of body mass and environment (tropical or temperate)

	Cd	H_2O_2	UV	PQ	MMS		Thapsigargin	Tunicamycin
	Intercept	Intercept	Intercept	Intercept	Intercept	Slope	Intercept	Intercept
No phylogeny								
Best model	Environment	Environment	None	Environment	Environment × body mass		Environment	Environment
AIC_c	**493.5**	**570.0**	**805.8**	**487.3**	**140.6**	NA	**394.9**	502.0
Temperate LD_{50}	58.5	137.9	1469.3	55.1	1.3	NA	18.0	53.8
Tropical LD_{50}	94.6	231.1	NA	61.8	1.9	NA	23.9	62.4
P	0.02*	0.01*	<0.0001*	0.6	0.1	NA	0.1	0.7
Brownian								
Best model	Environment	Environment	None	Environment	None		Environment	None
AIC_c	525.3	592.7	833.7	513.8	201.7	NA	306.5	576.7
Temperate LD_{50}	44.4	36.2	912.1	37.4	1.4	NA	17.0	64.0
Tropical LD_{50}	84.6	244.5	NA	54.6	NA	NA	18.1	NA
P	0.006*	<0.0001*	0.6	0.2	NA	NA	0.7	0.5
Pagel								
Best model	Environment	Environment	None	Environment	Environment		Environment	Environment
AIC_c	NA	**571.8**	**806.1**	489.2	151.7		**296.4**	**478.3**
Temperate LD_{50}	NA	112.5	1151.2	52.3	0.9	0.0007	16.0	57.5
Tropical LD_{50}	NA	224.2	NA	60.2	1.0	0.02	22.8	57.8
P	NA	0.004*	0.007*	0.6	0.9	0.0005*	0.08	0.9
Martins								
Best model	Environment	Environment	None	Environment	Environment × body mass		Environment	Environment
AIC_c	**494.3**	571.8	**805.8**	NA	**142.6**	NA	**295.6**	503.6
Temperate LD_{50}	57.1	134.5	1428.1	NA	1.3	NA	18.1	52.0
Tropical LD_{50}	93.1	231.3	NA	NA	1.9	NA	22.2	62.5
P	0.02*	0.007*	<0.0001*	NA	0.1	NA	0.2	0.6
Grafen								
Best model	Environment	Environment	None	Environment	Environment × body mass		Environment	Environment
AIC_c	**495.3**	572.0	**805.9**	**488.7**	**142.4**	NA	**296.9**	503.3
Temperate LD_{50}	56.1	137.9	1347.3	52.2	1.2	NA	18.0	48.6
Tropical LD_{50}	95.2	231.1	NA	60.6	1.9	NA	23.9	68.4
P	0.01*	0.01*	0.001*	0.6	0.07	NA	0.1	0.3

Shown for each evolutionary hypothesis is the best model from a suite of five candidate models, the corrected Akaike's information criterion (AIC_c) value of this best model, parameter estimates for the intercept (tropical and temperate) and slope (body mass), with associated *P*-values for differences between intercepts or slopes. Significant variables are emphasized with an asterisk and the evolutionary model within each stressor with lowest AIC_c is bold.
LD_{50}, dose that kills 50% of cells; NA, not applicable.

We should also note, however, that the AIC_c values of models incorporating phylogeny were never lower (i.e. better) than the model without phylogeny. In many cases, models accounting for phylogeny were within two AIC_c units (higher) of the best non-phylogeny model. Thus, accounting for the phylogenetic relationships among species did little to improve the fit of the models.

The particular species of tropical bird that had higher tolerance to chemical stressors varied with treatment, but some species uniformly had high tolerance to oxidative stress. The white-tipped dove, jacana, clay colored robin and mangrove swallow were the most tolerant when exposed to Cd or H_2O_2. When exposed to MMS and H_2O_2, the clay colored robin and the jacana were still two of the most tolerant species for this treatment.

DISCUSSION

We hypothesized that because tropical birds live a slow pace of life, their cells would be more resistant to chemical stress agents than cells from their temperate equivalents. Our data indicate that cells from tropical species have a higher LD_{50} after exposure to several forms of oxidative and non-oxidative stress. Thus, our study offers compelling evidence that the life history of a species influences not only whole-organism physiology, but also cellular attributes. Cells from tropical birds were more resistant to chemical injury than were cells from temperate birds.

The free radical theory of aging proposed by Harman (Harman, 1956) states that metabolic rate and longevity are linked by the amount of free radicals produced during aerobic respiration. Aging results from the accumulation of biological damage caused by the production of free radicals (Harman, 2001). To deal with these stresses, cells possess a network of defenses that include antioxidants, ion transporters and metal chelators, as well as repair systems for damaged cellular macromolecules such as DNA, lipids and proteins (Pacifici and Davies, 1991). One might suspect that the higher the rate of metabolism, the more free-radical production, but the production of free radicals in mitochondria is more complicated (Ricklefs and Wikelski, 2002; Harman, 2001; Barja, 2007). Birds have higher metabolic rates and longer lifespans, but have lower rates of ROS generation, than similarly sized mammals (Barja, 2007; Strecker et al., 2010). Thus, birds provide a unique study system to address how ROS production affects aging and cellular stress resistance processes (Pamploma et al., 1999).

Birds accumulate less ROS-induced damage than mammals, which, according to the free radical theory of aging, should lead to a longer lifespan (Ogburn et al., 1998; Harman, 2001; Strecker et al., 2010). However, how ROS production and the accumulation of damage to macromolecules are related to life-history differences in tropical and temperate birds remains unknown. ROS production by mitochondria is largely dependent on respiration rate, such that highly active mitochondria have high concentrations of ADP and

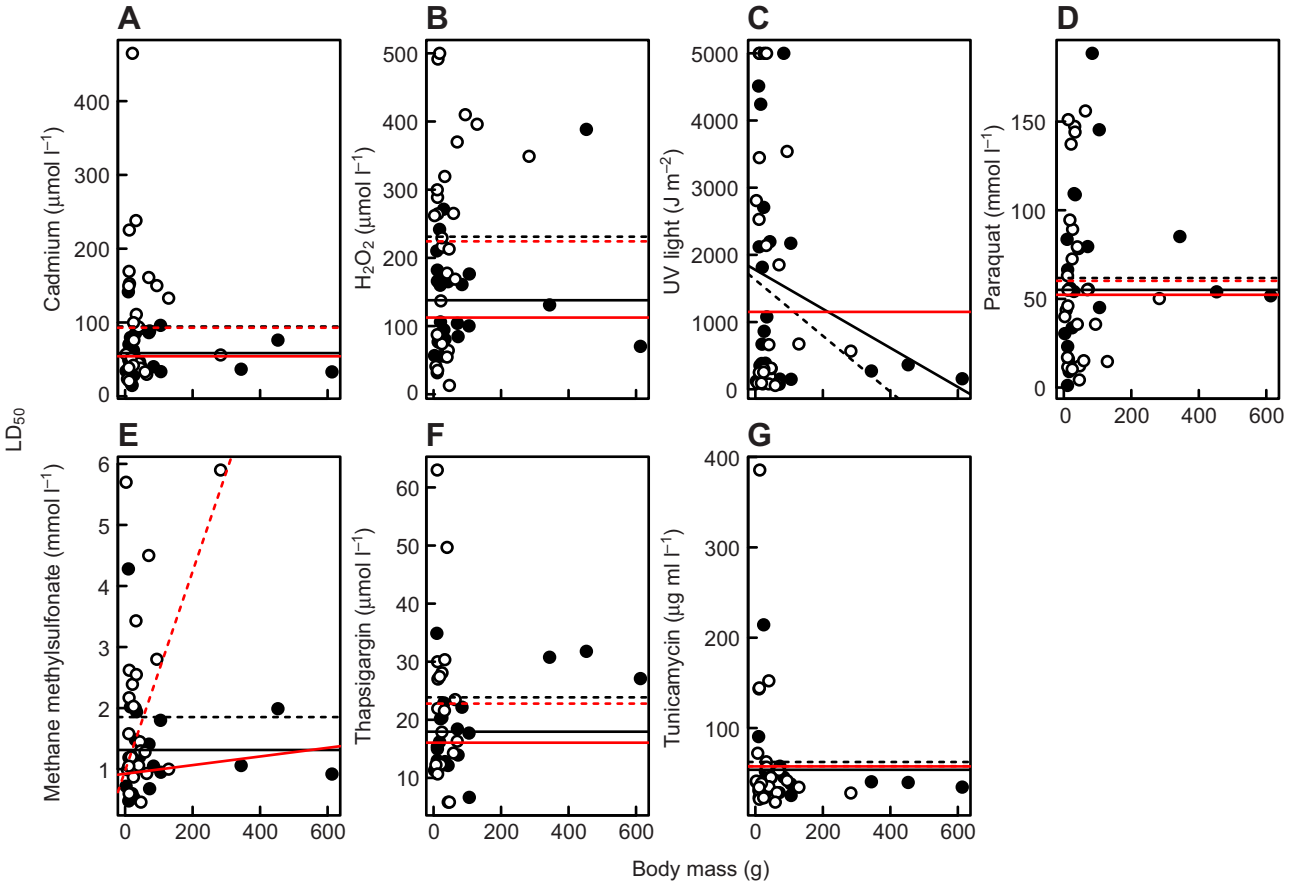

Fig. 1. The association between body mass and mean LD_{50} (the dose that killed 50% of cells) for each species in tropical (○) and temperate (●) birds. The lines show the outcome of a least square regression, where the dashed line represents the regression for tropical birds and the solid line the regression for temperate birds. We show fitted lines for the basic non-phylogenetic model in black and those for a model including Pagel's evolutionary hypothesis in red.

high rates of ATP synthesis, which results in a lower potential across the inner mitochondrial membrane (IMM); and when the potential across the IMM is low, almost no ROS are produced (Chance et al., 1979; Barja, 2007). In contrast, and controlling for the number of mitochondria, when cellular metabolism is low, the high potential across the IMM results in moderate ROS production (Chance et al., 1979; Cadenas and Boveris, 1980; Van Voorhies, 2004).

Tropical birds have a significantly lower basal and peak metabolic rate than temperate birds (Wiersma et al., 2007a; Wiersma et al., 2007b), which may translate into a higher production of damaging free radicals, because ROS production is highest when respiration rate is low. However, because tropical birds have high rates of survival (Ricklefs, 1997; Tieleman et al., 2006), they may have evolved efficient cellular mechanisms to regulate ROS-induced damage. By extension, the increased production of ROS in tropical birds should lead to increased oxidative stress defense mechanisms. Montgomery et al. found that the lifespan of birds was positively correlated with the antioxidants glutathione peroxidases and glutathione-*S*-transferases (Montgomery et al., 2012). These high antioxidant activities were hypothesized to protect against the production of hydroxyl radicals from hydrogen peroxide *via* the Fenton reaction. Thus, long-lived bird species, such as tropical birds, may utilize this mechanism to guard against the production of hydroxyl radicals, or it may be an adaptive response to inherently high levels of oxidative stress associated with high production rates of hydrogen peroxide and lipid hydroperoxides (Montgomery et al., 2012).

The fact that temperate birds have a higher basal metabolism than do tropical birds may suggest that the former have higher ROS production by their mitochondria (Perez-Campo et al., 1998); the prevailing view is that ROS production is higher in short-lived species (such as temperate birds) and that these species should show high levels of tissue antioxidants in order to combat their high rates of ROS production (Barja et al., 1994; Herrero and Barja, 1997; Lopez-Torres et al., 1993; Perez-Campo et al., 1994). At least in birds, it has recently been shown that the antioxidant capacity has a complex pattern relative to longevity (Cohen et al., 2008), whereby higher antioxidant levels were generally characteristic of 'fast-paced' life-history traits such as rapid development, low survival, smaller body size, larger clutch size and higher mass-specific metabolic rate. Thus, convincing *a priori* predictions about how cells of tropical and temperate birds might resist oxidative and non-oxidative stress are difficult to make.

ROS production and antioxidant defenses alone are not the only factors that could alter oxidative stress resistance and lifespan. The level of susceptibility of lipids in membranes to oxidative damage may also be important. The 'membrane pacemaker theory of aging' suggests that phospholipids vary in their susceptibility to peroxidation, leading to the production of secondary lipid-based ROS (Hulbert et al., 2007). A possible physiological mechanism that would allow birds to have high metabolic rates and long lifespans is a decrease in the number of double bonds in membrane phospholipids. Unsaturated fatty acids are sensitive to free radical damage, thus low double bond content should be advantageous by

decreasing the sensitivity of tissues to lipid peroxidation; this would be crucial in mitochondrial membranes as they are the major source of ROS (Pamplona et al., 1999). Pamplona et al. noted that the degree of unsaturation of fatty acids and the sensitivity of lipid peroxidation of liver and heart mitochondria are lower in the long-lived pigeon than in the short-lived rat, although they have similar metabolic rates (Pamplona et al., 1996; Pamplona et al., 1999). The susceptibility of fatty acids to free radical damage increases exponentially as a function of the number of double bonds per fatty acid molecule. Also, lipid peroxidation products are known to damage nearby macromolecules including proteins, a process that has consequences for aging (Pamplona et al., 1999). These ideas suggest that tropical birds have a low degree of fatty acid unsaturation in their membranes, which might protect them from oxidative damage (Pamplona et al., 1999; Montgomery et al., 2011).

In summary, we found that primary cell lines derived from tropical birds are more resistant to some cellular stressors compared with cells from their temperate counterparts. The physiological mechanism that allows tropical birds to be more resistant to these stressors is unclear; however, an increased resistance to ROS damage seems to be an important pathway to allow these birds the capacity to occupy the 'slow pace of life' part of the spectrum.

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AUTHOR CONTRIBUTIONS

A.G.J. drafted the article and compiled the data. J.M.H. grew the bird cell lines and gathered the data. A.G.J. and J.M.H. contributed to this paper equally. S.Q. analyzed the data gathered. J.B.W. collected all the birds included in this study and was responsible for designing the study.

COMPETING INTERESTS

No competing interests declared.

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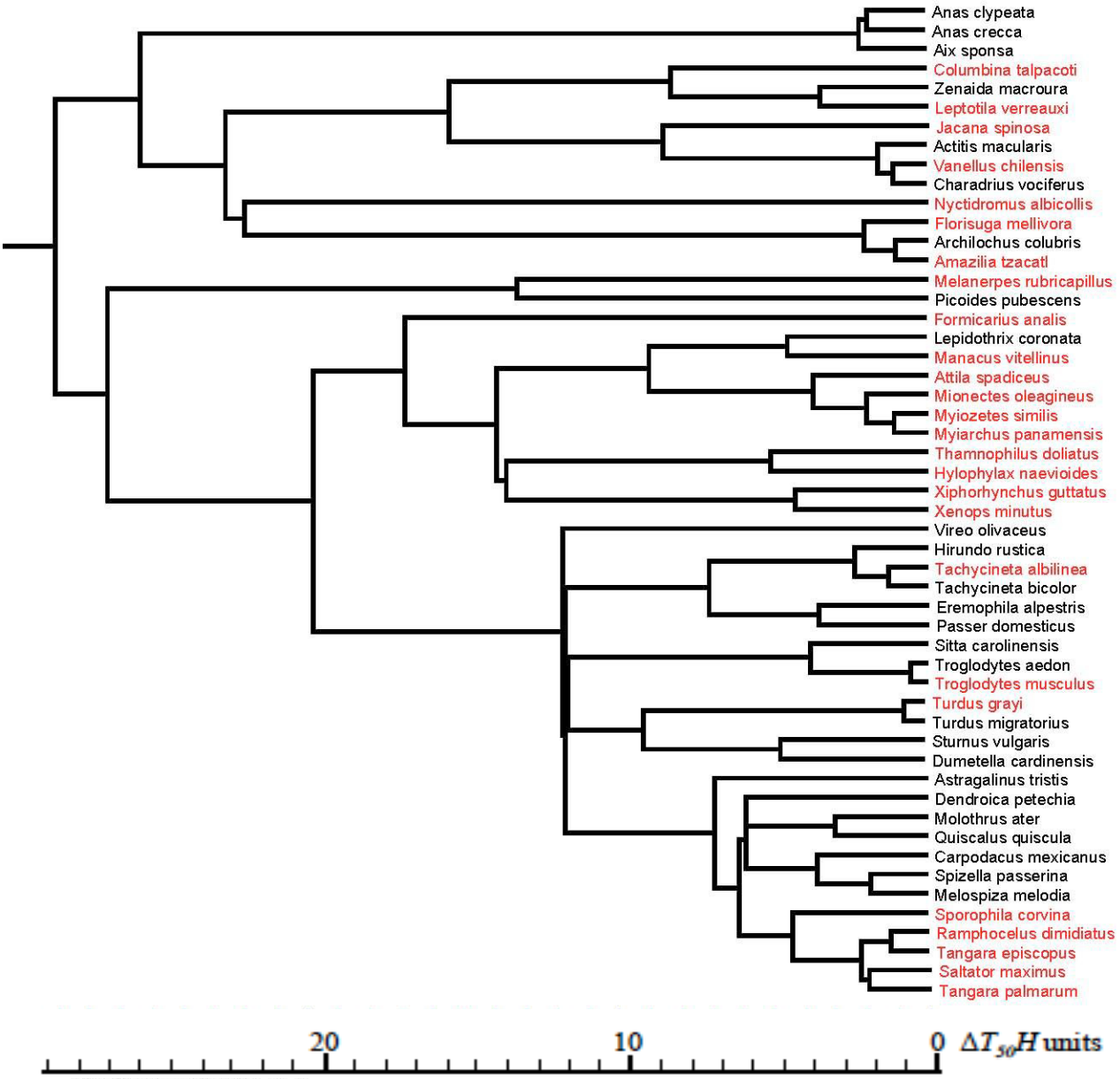

Fig. S1. Phylogenetic tree of 52 tropical and temperate bird species. Phylogenetic relationships are based on information assembled by Johnson and Sorenson (Johnson and Sorenson, 1999), Klicka et al. (Klicka et al., 2000), Yuri and Mindell (Yuri and Mindell, 2002) and Boyd (Boyd, 2011). Branch lengths were derived from Sibley and Ahlquist (Sibley and Ahlquist, 1990) and shown in units of difference in melting temperatures of bonded DNA strands of different species. Tropical species are designated in red lettering, and temperate species in black lettering.

Table S1. The five candidate models tested with AIC for each LD_{50} treatment

Model	Predictor variable(s)	Description
1	None	No effect of environment or covariate
2	Environment	Effect of environment only
3	Covariate	Effect of covariate only (body mass)
4	Environment + covariate	Additive model
5	Environment × covariate	Including an interaction