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Free radicals run in lizard families

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In the ageing individual, the production of reactive oxygen species (ROS) accelerates with cell senescence. Depending on the heritability of the underlying processes that determine net ROS levels, this may influence ageing *per se* and its evolutionary direction and rate of change. In order to understand the inheritance and evolution of net ROS levels in free-ranging lizards, we used flow cytometry together with ROS-sensitive fluorogenic probes to measure ROS in lizard blood cells. We measured basal levels of (i) non-specific ROS (superoxide, singlet oxygen, H_2O_2 and peroxynitrite), (ii) superoxide specifically and (iii) superoxide after CCCP treatment, which elevated ROS production in the mitochondria. The cumulative level of non-specific ROS was higher in adults than juveniles and superoxide level showed high heritability and variability among families. We suggest that the evolution of ROS dynamics may be ROS species specific and perhaps depend on the relative degree of uni- or biparental inheritance of ROS main regulatory pathways.

Keywords: reactive oxygen species; mitochondrial inheritance; heritability

1. INTRODUCTION

An inevitable result of aerobic metabolism is the build-up of reactive oxygen species (ROS) in cells as a by-product of the capture of chemical energy (ATP) by the electron transport chain (ETC). This may have major consequences for viability since ROS accelerates mitochondrial decay, which further reduces ETC efficiency in a vicious cycle of ROS production, and may result in ageing of the organism (Harman 1957; Shigenaga *et al.* 1994; Finkel & Holbrook 2000; Barja 2004). Therefore, understanding the underlying genetic principles of the evolution of ROS generation and depletion is important for evaluating alternative hypotheses for the evolution of senescence (Shigenaga *et al.* 1994).

Although great progress has recently been made towards explaining ROS regulation from a genetic and proximate perspective, such as in terms of circulating levels of vitellogenin as an ROS scavenger (Seehuus *et al.* 2006; Olsson *et al.* in press), very little is still known about its natural variation and inheritance. Without this, we cannot make inferences on how potentially ROS-induced ageing evolves in free-ranging organisms, or predict its downstream consequences for longevity and life-history evolution.

To analyse ROS, we used flow cytometry in combination with two probes that freely diffuse into cells, accumulate within the mitochondria and become fluorescent when oxidized by specific ROS (MitoSOX Red identifies superoxide and DHR (dihydrorhodamine 123) identifies singlet oxygen, superoxide, H_2O_2 and peroxynitrite, hereafter referred to as 'non-specific ROS'; Vowells *et al.* 1995; Spence 2005). We analysed 50 000 blood cells from wild-caught female painted dragon lizards (*Ctenophorus pictus*) and their offspring to estimate the heritability of the propensity of the ETC in the mitochondria to generate different ROS species. Superoxide, for example, is a direct by-product of the ETC and its production is strongly influenced by maternally encoded mitochondrial genes (Wallace & Lott 2002). Peroxynitrite levels, however, depend on superoxide's reaction with nitric oxide (NO), made available by NO synthase, the production of which is encoded by nuclear genes (Wallace & Lott 2002). We also used the chemical CCCP (cyanide 3-chlorophenylhydrazone) to manipulate ROS production. CCCP administration mildly uncouples mitochondria, which increases electron flow through the ETC and several studies report a concomitant decrease in mitochondrial ROS at CCCP concentrations of 1–10 µM, whereas lower and higher concentrations may lead to increased ROS production in mammalian cells (Alberts *et al.* 1994; Gilmore & Wilson 1999). In our experiments on lizards, CCCP treatment leads to an approximate tripling of superoxide fluorescence counts (see §3). We took advantage of this to assess the heritability of two aspects of ROS regulation, with and without dissipation of the electron–proton gradient (which results in one normal ROS estimate and one estimate outside of its normal range). We also demonstrate elsewhere that females that lay relatively larger clutches also have relatively lower levels of ROS (Olsson *et al.* in press), most likely owing to higher levels of circulating vitellogenin that may act as an ROS scavenger (Seehuus *et al.* 2006). We therefore also include a yolk manipulation experiment to assess the risk of having an important maternal effect influencing our heritability estimates of ROS.

2. MATERIAL AND METHODS

Female Australian painted dragon lizards (*C. pictus*) were caught by noose or hand at Yathong Nature Reserve, New South Wales (145°35′, 32°35′) and were brought back to the University of Wollongong. The lizards were kept singly in cages (330×520×360 mm), on a 12 : 12 hour light regime (light : dark), with a spotlight to allow thermoregulation and were fed crickets and mealworms every second day. Eggs were incubated in moist vermiculite (mixed with tap water in a 7 : 1 ratio) at 30°C (±2.5°C) within 4 hours of laying until hatching (approx. 60 days). Lizards were weighed to the nearest 0.1 g and measured to the nearest 1.0 mm.

Assessments of cross-generational relationships, such as heritabilities, are potentially confounded by parental effects (Falconer & Mackay 1996). Therefore, we made use of allometric engineering (Sinervo & Huey 1990), during which yolk is extracted from the egg at oviposition (by suction with a syringe and using a 0.5× 16 mm^2 needle) and compared with a control group in which the egg shell is only punctured and the needle inserted and withdrawn without yolk removal. We used 33 offspring from 16 different females for this experiment (19 yolk reduced, 14 controls). On

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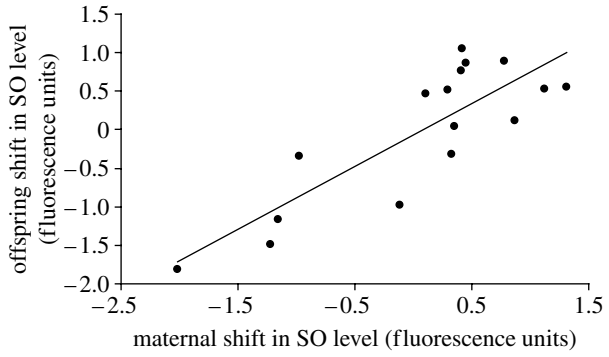

Figure 1. The increase in superoxide level (SO) above basal level subsequent to mitochondrial uncoupling is strongly heritable, suggesting that parent–offspring similarity in the baseline level of SO is caused by maternal (mitochondrial genetic) effects ($F_{1,14}=33.6$, $p<0.0001$).

average, 0.196 g (±0.004 s.e.) yolk was removed from yolk manipulated eggs, compared with 0.0165 g (±0.007 s.e.) from controls (due to leakage of albumin from the punctured egg shell). This represents an 11.9-fold difference in egg mass reduction between treatments and controls, and represents an approximately 25% reduction of egg mass in the treatment group (1.29 g, ±0.03 s.e.). This difference between treatments and control is highly significant ($t=21.9$, $p<0.0001$).

Peripheral blood (100 µl, adults; 10 µl, young) was sampled in the morning of flow cytometry (2 days, November 2005) with a glass capillary from vena angularis (in the corner of the mouth). Cells were analysed with no additions (unstained control), 0.1 mM dihydrorhodamine 123 (DHR; Molecular Probes, Invitrogen, USA), 5 µM MitoSOX Red (MR; Molecular Probes, Invitrogen) or 5 µM MR plus 10 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma, Sydney, Australia). Flow cytometry was performed using a Becton Dickinson LSR II, with excitation at 488 nm and emitted fluorescence collected using bandpass filters of 515±10 nm (DHR) and 575±13 nm (MR). Data were acquired and analysed using FACSDiva v. 4.0.1 and CellQuest Pro v. 5.1.1 software (Becton Dickinson, Sydney, Australia), respectively. For further details, see Olsson *et al.* (in press).

Genetic effects on ROS variation were calculated in two ways (sample sizes varies between different experiments and are given in association with corresponding statistical tests in §3): (i) as heritabilities in parent–offspring regressions (mean offspring ($n=47$) trait value regressed on maternal trait value ($n=18$)) with ROS estimates standardized by sampling date (they were higher at our second sampling event), and (ii) in a full-sib analysis, we assessed the effect of maternal identification number on ROS variation with sampling date and offspring age (older hatchlings had more ROS) as covariates (to avoid any potential bias from comparing field-caught mothers with laboratory-reared offspring). The analysis was performed with the REML option (Proc Mixed, SAS) followed by likelihood ratio tests. The painted dragon is a near-annual species (less than 10% survive to a second year; Olsson *et al.* in press). Thus, all females are approximately 1 year old and, hence, variation in female age is unlikely to drive a parent–offspring relationship in ROS variation in a predictable way.

3. RESULTS

Offspring age (mean 14.9 days±8.8 s.d., minimum 5, maximum 42) was positively correlated with non-specific ROS level ($r=0.50$, $p=0.035$, $n=16$), but not with corresponding estimates of superoxide ($r=-0.21$, $p>0.41$). Non-specific ROS levels differed between adult females and juveniles (1.10±0.16 s.e. versus −0.48±0.11 s.e., in adults versus juveniles, respectively; *t*-test; $t=8.16$, $p<0.00001$, d.f.=32). The corresponding *t*-tests for superoxide with and without CCCP treatment showed corresponding differences (mean superoxide for hatchlings versus mothers, 11.6±0.64 s.e., $n=47$ and 14.5±1.15 s.e., respectively; $t=2.31$, d.f.=30.8 (Satterthwaites' approximation), $p=0.024$; mean superoxide+CCCP, 25.2±0.86 s.e., and 29.3±1.59, respectively; $t=2.48$, d.f.=30.8, $p=0.016$).

The heritability of non-specific ROS did not differ significantly from zero ($F_{1,15}=0.03$, $p=0.88$). The parent–offspring regression of basal superoxide level was statistically significant ($F_{1,14}=8.3$, $p=0.012$, $R^2=0.37$, estimated heritability=0.45±0.16 s.e.), and even more pronounced subsequent to mitochondrial uncoupling by CCCP ($F_{1,14}=13.1$, $p=0.003$, $R^2=0.48$; estimated heritability=0.54±0.15). We then subtracted basal superoxide level from induced level to specifically isolate the effects of mitochondrial uncoupling for analysis. This revealed an even higher heritability (figure 1; $F_{1,14}=33.6$, $p<0.0001$, $R^2=0.71$; estimated heritability=0.82±0.14 s.e.).

Our full-sib analysis showed that, when we removed the variance from offspring age and date of measurement on ROS levels, all three of our ROS estimates showed significant family effects (non-specific ROS: $h^2=0.49$, LR=5.1, d.f.=1, $p<0.025$; superoxide: $h^2=0.55$, LR=6.3, d.f.=1, $p<0.025$; superoxide at CCCP treatment: $h^2=0.73$, LR=9.7, d.f.=1, $p<0.005$).

The results of our allometric engineering experiment showed no effect of yolk manipulation on the levels of offspring superoxide ($F_{1,15}=0.01$, $p=0.91$), whereas the effect of maternal identity was significant (likelihood ratio test: LR=5.5, $p=0.019$). The corresponding treatment effect on non-specific ROS showed a similar pattern (treatment $F_{1,15}=1.81$, $p=0.20$; maternal identity LR=3.9, $p=0.048$). Thus, our manipulation of yolk level shows no effect on the levels of offspring ROS and, hence, variation in maternal yolk investment cannot explain any covariation between maternal and offspring ROS.

4. DISCUSSION

We show that the summed effect of processes regulating net cellular ROS levels that must have been under selection for considerable evolutionary time can have high heritable variation. However, the level of heritability may vary among ROS species and our allometric engineering experiment shows that these results are not an effect of differential yolk investment by females. However, we acknowledge that other potential maternal effects, such as yolk composition, may contribute to parent–offspring correlations, and that this may constitute a component in our heritability estimate (Falconer & Mackay 1996). Since the production of different ROS species depends on regulatory processes partly encoded by the non-recombined, maternally inherited mitochondrial genome, and partly by a sexually recombined, biparentally inherited genome, heritability and concomitant response to selection may differ among ROS species. Here we show that a property as fundamental as net cellular ROS level can still have considerable heritable variation depending on ROS species. Recent studies of kestrel nestlings (*Falco tinnunculus*) showed a negative relationship between age and oxidative stress (Constantini *et al.* 2006), and that a significant part of the variance in reactive oxygen metabolites could be explained by genetic factors as revealed by a

cross-fostering experiment (Constantini & Dell'Omo 2006). Thus, this agrees with the results presented here. Furthermore, the extremely high heritability of superoxide in combination with CCCP uncoupling ($h^2 = 0.82$) suggests (i) strong family effects on how this molecule affects the proton gradient (perhaps through the transport of protons across the membrane (Alberts *et al.* 1994) and (ii) that perhaps the underlying genes that encode this process may rarely be recombined (if mitochondrial), which constrains the rate at which selection can act on the evolution of senescence trajectories (Birky 1994; Rand 2001). In spite of the high heritability of superoxide levels, their encoding genes can only evolve if unconstrained by genetic correlations with traits under selection, which is typically not the case of traits with uniparental inheritance (Birky 1994; Rand 2001). Thus, the lack of independence among mitochondrial genes means that the efficacy of selection is reduced compared with nuclear genes (Birky 1994; Rand 2001; Bazin *et al.* 2006).

An important way towards understanding the evolution of net ROS levels may be to investigate heritability and genetic correlations between ROS and ETC traits, and assess survival and longevity in the wild of individual animals that differ in these aspects. For many of these traits, cell biologists have developed high-resolution analytical tools (e.g. to assess mitochondrial density and ROS-specific levels), but they still remain uninvestigated by evolutionary biologists from a perspective of evolvability.

This research was approved by the Animal Ethics Committee, University of Wollongong (permit number AE04/03-05).

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Alberts, B., Bray, D., Lewis, J., Raft, M., Roberts, K. & Watson, J. D. 1994 *Molecular biology of the cell*, pp. 728–729, 3rd edn. New York, NY: Garland Publishing.

Barja, G. 2004 Ageing in vertebrates, and the effect of caloric restriction: a mitochondrial free radical production–DNA damage mechanism? *Biol. Rev.* **79**, 235–251. (doi:10.1017/S1464793103006213)

Bazin, E., Glémin, S. & Galtier, N. 2006 Population size does not influence mitochondrial genetic diversity in animals. *Science* **312**, 570–572. (doi:10.1126/science.1122033)

Birky Jr, C. W. 1994 Uniparental inheritance and mitochondrial and chloroplast genes: mechanisms and evolution. *Proc. Natl Acad. Sci. USA* **92**, 11 331–11 338. (doi:10.1073/pnas.92.25.11331)

Constantini, D. & Dell'Omo, G. 2006 Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B* **176**, 575–579. (doi:10.1007/s00360-006-0080-0)

Constantini, D., Casagrande, S., De Filipis, S., Brambilla, G., Fanfani, A., Tagliavini, J. & Dell'Omo, G. 2006 Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B* **176**, 329–337. (doi:10.1007/s00360-005-0055-6)

Falconer, D. S. & Mackay, T. F. S. 1996 *Introduction to quantitative genetics*, 4th edn. New York, NY: Longman Scientific & Technical.

Finkel, T. & Holbrook, N. J. 2000 Oxidants, oxidative stress and the biology of ageing. *Nature* **400**, 239–247. (doi:10.1038/35041687)

Gilmore, K. & Wilson, M. 1999 The use of Chloromethyl-X-Rosamine (Mitotracker Red) to measure loss of mitochondrial membrane potential in apoptotic cells is incompatible with cell fixation. *Cytometry* **36**, 355–358. (doi:10.1002/(SICI)1097-0320(19990801)36:4<355::AID-CYTO11>3.0.CO;2-9)

Harman, D. 1957 Ageing: a theory based on free radical and radiation chemistry. *J. Gerontol.* **2**, 298–300.

Olsson, M., Wilson, M., Uller, T., Mott, B. & Isaksson, C. In press. Variation in levels of reactive oxygen species is explained by maternal identity, sex, and relative clutch size in a lizard. *Naturwissenschaften*.

Rand, S. 2001 The unit of selection on mitochondrial DNA. *Annu. Rev. Ecol. Syst.* **32**, 415–448. (doi:10.1146/annurev.ecolsys.32.081501.114109)

Seehuus, S.-C., Norberg, K., Gimsa, U., Krekling, T. & Amdam, G. 2006 Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl Acad. Sci. USA* **103**, 962–967. (doi:10.1073/pnas.0502681103)

Shigenaga, M. K., Hagen, T. M. & Ames, B. N. 1994 Oxidative damage and mitochondrial decay in ageing. *Proc. Natl Acad. Sci. USA* **91**, 10 771–10 778. (doi:10.1073/pnas.91.23.10771)

Sinervo, B. & Huey, R. B. 1990 Allometric engineering: an experimental test of the causes of interpopulational differences in performance. *Science* **248**, 1106–1109. (doi:10.1126/science.248.4959.1106)

Spence, M. 2005 *The handbook. A guide to fluorescent probes and labeling technologies*, 10th edn. Eugene, OR: Invitrogen Molecular Probes.

Vowells, S., Sekhsaria, S., Malech, H., Shalit, M. & Fleisher, T. 1995 Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. *J. Immunol. Methods* **178**, 89–97. (doi:10.1016/0022-1759(94)00247-T)

Wallace, D. C., Lott, M. T., 2002 Mitochondrial defects of common diseases. In *The genetic basis of common diseases* (eds R. A. King, J. I. Rotter & A. G. Motulsky), pp. 975–988, 2nd edn. New York, NY: Oxford University Press.