Invited reply

Free radicals run in lizard families without (and perhaps with) mitochondrial uncoupling

Criscuolo & Bouillaud's (2009, hereafter 'CB') interesting comment on our recent papers on lizard reactive oxygen species (ROS) biology (Olsson *et al.* 2008a,b) is a nice demonstration of the complex mechanisms underlying the production and elimination of ROS (and other reactive molecules) and what makes them manifest at the net levels we measure. Before we respond to CB's central focus—ROS effects of mitochondrial uncouplers—we would like to point out that:

- (i) our paper (Olsson *et al.* 2008a) contains no information on mitochondrial uncoupling; it is a straightforward demonstration using flow cytometry and fluorogenic probes that carotenoids do not depress ROS at normal electron transport chain (ETC) activity and
- (ii) more importantly, we demonstrate (Olsson *et al.* 2008b) that unmanipulated, basal level of superoxide (i.e. net effect of production and antioxidation of superoxide, SO) has significant heritability ($h^2 = 0.45 \pm 0.16$, s.e., $p = 0.012$). Thus, chemical interference with ETC function is not necessary for detecting heritability for SO and, in this regard, CB's title is somewhat misleading. Considering that SO production is strongly influenced by maternally encoded mitochondrial genes (i.e. with very limited, or non-existent, recombination), any other result would, in fact, have been remarkable although, to our knowledge, Olsson *et al.* (2008b) were the first to test this.

We agree with the underlying logic of CB's take-home message—that mitochondrial uncoupling reduces SO production and prolongs lifespan (the 'uncoupling to survive' hypothesis). The biochemical mechanism by which carbonyl cyanide 3-chlorophenyl hydrazone (CCCP), usually referred to as an 'artificial uncoupler', elevates ROS production was never a target in either of our studies. Furthermore, chemical uncouplers such as CCCP are known to both decrease (Skulachev 1996; Korshunov *et al.* 1997; Breitenbach *et al.* 2003; Kadenbach 2003) and increase (Stöckl *et al.* 2007; Olsson *et al.* 2008b) ROS production in a dose-dependent and exposure-duration manner. At low doses, CCCP favours the entrance of protons into the mitochondrial matrix

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(uncoupling activity), whereas at high doses (more than or equal to 10 μM), CCCP inhibits respiration by acting at the Q-cycle, which triggers a higher production of SO. In yeast, another artificial uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, increases ROS production at 5 μM with a subsequent reduction in cell lifespan (Stöckl *et al.* 2007). Thus, among-taxon variation in the dose-dependent effects of artificial uncouplers on ROS levels are apparent. Therefore, the concentration of CCCP that CB refers to, at which the ETC is fully uncoupled, and above which respiration starts to become inhibited, is likely to be taxon specific, and, to the best of our knowledge, no reptile species has been characterized in this regard. Thus, neither CB nor Olsson *et al.* know what level of uncoupling Olsson *et al.* (2008b) achieved on the 'mild' and 'full' uncoupling scale (stating it was 'mild' may have been misleading, our apology).

Importantly, however, the effect of CCCP elevation on ROS in our study is itself strongly heritable, suggesting that regardless of biochemical underpinning, this effect is worth examination since it would respond to selection and may contribute to ETC evolution. CB concludes that our CCCP effect on SO net levels, and its corresponding strong heritability, may be due to either adverse effects of CCCP on antioxidant production or resistance of respiratory chain complexes to CCCP inhibitory impact. We agree that these potential contributors to the CCCP-elevated ROS levels remain unexplored. Our interest was primarily to, for the first time, put ROS production under the control of the experimenter in work on ETC and 'ROS effects evolution', not to identify the mechanism as to *how* ROS was manipulated.

The main conclusions from this exchange are: (i) net SO levels are heritable in our lizard model, with or without CCCP treatment ($h^2 = 0.45$ versus 0.54), (ii) artificial uncouplers should be referred to with greater care when their specific biochemical action is unclear (hence, protecting hypotheses such as 'uncoupled to survive'), and (iii) biochemical tools that allow researchers to manipulate ROS production (and antioxidation) are highly desirable, since the heritability of their effects may show what mechanisms are conserved and which ones show variation with the capacity to evolve.

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