

Oxidative shielding and the cost of reproduction

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ABSTRACT

Life-history theory assumes that reproduction and lifespan are constrained by trade-offs which prevent their simultaneous increase. Recently, there has been considerable interest in the possibility that this cost of reproduction is mediated by oxidative stress. However, empirical tests of this theory have yielded equivocal support. We carried out a meta-analysis to examine associations between reproduction and oxidative damage across markers and tissues. We show that oxidative damage is positively associated with reproductive effort across females of various species. Yet paradoxically, categorical comparisons of breeders *versus* non-breeders reveal that transition to the reproductive state is associated with a step-change reduction in oxidative damage in certain tissues and markers. Developing offspring may be particularly sensitive to harm caused by oxidative damage in mothers. Therefore, such reductions could potentially function to shield reproducing mothers, gametes and developing offspring from oxidative insults that inevitably increase as a consequence of reproductive effort. According to this perspective, we hypothesise that the cost of reproduction is mediated by dual impacts of maternally-derived oxidative damage on mothers and offspring, and that mothers may be selected to diminish such damage. Such oxidative shielding may explain why many existing studies have concluded that reproduction has little or no oxidative cost. Future advance in life-history theory therefore needs to take account of potential transgenerational impacts of the mechanisms underlying life-history trade-offs.

Key words: ageing, fetal programming, oxidative shielding hypothesis, oxidative stress, life-history trade-off, reactive oxygen species.

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I. INTRODUCTION

The cost of reproduction is a cornerstone of life-history theory. High levels of investment in current reproduction can decrease survival to the next reproduction, or reduce future reproductive success (Williams, 1966; Stearns, 1992). While such trade-offs have been extensively documented in animals, the underlying mechanisms remain unclear (Isaksson, Sheldon & Uller, 2011; Selman *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014). Theoretically, reproduction is costly because it entails increased levels of oxidative damage (Costantini, 2008; Dowling & Simmons, 2009; Monaghan, Metcalfe & Torres, 2009). Reproduction incurs elevated metabolic rate, and in turn, this is predicted to result in increased generation of reactive oxygen species (ROS) which are mainly a byproduct of ATP synthesis in mitochondria. ROS are highly unstable and can cause oxidative damage to DNA, lipids and proteins. A state of oxidative stress can arise if ROS production overwhelms the body's antioxidant defences or repair mechanisms, resulting in increased levels of oxidative damage, and loss of cell homeostasis and function. Oxidative damage is thought to be an important mechanism underlying the gradual deterioration of bodily function that defines ageing (Halliwell & Gutteridge, 1999), and therefore could explain the cost of reproduction (Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014).

Empirical tests of this theory have so far yielded equivocal support, which recent reviews have attributed to limitations in study design (Isaksson *et al.*, 2011; Selman *et al.*, 2012; Stier *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014). For example, many early studies failed to measure markers of oxidative damage, instead reporting only measures of antioxidant protection in order to infer an individual's oxidative state. It is now generally agreed that this approach is insufficient, because levels of antioxidants may both increase and decrease during oxidative stress; markers of oxidative damage must be measured in order to determine oxidative stress (Selman *et al.*, 2012). Several other explanations for the inability to detect an oxidative cost of reproduction have been proposed, including a failure to measure an appropriate range of tissues or markers of oxidative damage, a failure to study animals under naturalistic levels of resource limitation, or in the wild, a failure to take into account variation in the timing of measurements, and the possibility that parents may pre-emptively increase their antioxidant

defences to avoid somatic damage (Garratt *et al.*, 2011, 2013; Oldakowski *et al.*, 2012; Selman *et al.*, 2012; Stier *et al.*, 2012; Metcalfe & Monaghan, 2013). In addition, it has recently been suggested that an important limitation of existing studies is a failure to experimentally manipulate reproductive effort, leaving open the possibility that individuals may modulate current reproductive effort to maximise their lifetime reproductive success (Metcalfe & Monaghan, 2013). Such modulation is likely to vary according to an individual's quality or access to resources and could give rise to positive correlations between life-history traits (van Noordwijk & De Jong 1986), and thus obscure the oxidative cost of reproduction (Metcalfe & Monaghan, 2013). Finally, it has been suggested that conceptual models of an oxidative cost of reproduction are too simplistic, not least because ROS production is not directly proportional to metabolic rate and in some circumstances can decrease as metabolic rate increases (Selman *et al.*, 2012; Speakman & Garratt, 2014).

As a result of these concerns, there is a growing consensus that we need additional, carefully designed experiments that may conclusively test whether reproduction incurs increased oxidative damage (Isaksson *et al.*, 2011; Selman *et al.*, 2012; Stier *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014). However, at the same time, a handful of studies have recently emerged reporting the opposite pattern to that predicted: a significant decrease in oxidative damage in breeders, at least in some tissues and markers (Garratt *et al.*, 2011, 2012, 2013; Oldakowski *et al.*, 2012; Yang *et al.*, 2013; Xu *et al.*, 2014), while paradoxically showing that animals expending greater reproductive effort have raised levels of oxidative damage (Garratt *et al.*, 2011; Stier *et al.*, 2012; Yang *et al.*, 2013; Speakman & Garratt, 2014; Xu *et al.*, 2014). Despite any failings in the design of existing studies, why oxidative damage may become both decreased and increased in reproducing animals requires explanation.

II. AIMS

Faced with accumulating data from different sources that report conflicting or inconsistent results, meta-analysis is a powerful tool for synthesising research evidence across independent studies (Koricheva, Gurevitch & Mengersen, 2014). We carried out a meta-analysis, with the aim of examining whether there are consistent associations between reproduction and oxidative damage. Our analysis was restricted to females

of homeothermic species, for which the vast majority of data are available. In line with existing theoretical paradigms, we hypothesised that individuals expending greater reproductive effort have higher levels of oxidative damage, and reproducing individuals have higher levels of oxidative damage compared with non-reproducing individuals (reviewed by Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014). Based on the results of this analysis we propose a new hypothesis to explain why levels of oxidative damage are often decreased in breeders, while positive correlations between reproductive effort and oxidative damage are also observed.

III. MATERIALS AND METHODS

(1) Literature search and criteria for inclusion in the meta-analysis

Data for the meta-analysis were collated from the literature and from additional unpublished sources. The search was initiated using recent reviews (Selman *et al.*, 2012; Stier *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014), followed by additional searches using *Web of Science* and *Google Scholar* up to 15 May 2014. We used the search terms ‘reproduction’, ‘reproductive effort’, ‘oxidative damage’, ‘oxidative stress’ and their combinations. These searches yielded 1672 articles (including duplicate results); based on a reading of abstracts alone this list was reduced to 44 studies, which were read in full. The vast majority of studies of relationships between reproduction and oxidative damage have reported data for homeothermic species, and for females only. Therefore, we restricted our search to females of homeothermic species. We sought data for two types of analysis. First, we were interested to test the hypothesis that individuals expending greater reproductive effort have higher levels of oxidative damage (i.e. an analysis across females that have all reproduced, but have variable numbers or weights of offspring). For this analysis we collated data for the association between reproductive effort (i.e. clutch, brood or litter size; litter mass; or the proportion of eggs that hatched) and oxidative damage. Second, we were interested to test the hypothesis that levels of oxidative damage are higher in reproductive than in non-reproductive individuals; here we collated data for categorical comparisons of breeders *versus* non-breeders from the same populations or social groups.

We collated data for five markers which are commonly reported as indicators of oxidative damage: malondialdehyde, reactive oxygen metabolites, protein carbonyls, oxidised glutathione, and protein thiols (Halliwell & Gutteridge, 1999; Monaghan *et al.*, 2009; Selman *et al.*, 2012). Malondialdehyde is a marker of

lipid peroxidation which is quantified using either high-performance liquid chromatography (HPLC) or spectrophotometry; the latter method has lower specificity and accuracy than HPLC, but there is no reason to expect this to bias the results of the meta-analyses. Reactive oxygen metabolites are hydroperoxides, generated mainly as a result of lipid peroxidation. Protein carbonyls are produced when protein side chains become oxidised, and are therefore a marker of protein oxidation. Oxidised glutathione is a marker of redox status, and in some instances oxidative stress. The above markers were predicted to increase with reproduction. In addition, we collated data for protein thiols, groups on proteins that are susceptible to oxidation and are therefore a marker of protein oxidation. Reproduction is predicted to diminish levels of protein thiols, but for the purposes of the meta-analysis we reversed the sign of these data so all five markers of oxidative damage were predicted to increase with reproduction. This enabled us to calculate combined effect sizes across tissues and/or markers (see Section III.2). Data were available for blood plasma, serum and erythrocytes, skeletal muscle, liver, kidney, heart, and ovarian follicles (see online File S1, Tables S1 and S2).

If samples were collected at different times during reproduction, we used data for the sampling point that represented peak reproductive effort (peak lactation rather than weaning; Upreti, Chaki & Misro, 2002). In two studies of cooperatively breeding bird species, data were available for the period just prior to laying, during offspring care, and/or at completion of breeding (van de Crommenacker, Komdeur & Richardson, 2011; Cram, Blount & Young, 2015). In such species, subordinate ‘helpers’ often contribute to nestling care, and sometimes also engage in independent breeding attempts. Therefore in order to compare breeders against individuals that were functionally non-reproductive, we used data collected just prior to laying for dominant females and non-reproductive subordinates. For studies that experimentally manipulated a variable, i.e. ambient temperature, diet, or radiofrequency radiation (RF) exposure, in addition to measuring reproduction, we used data for the control group [i.e. room temperature (21°C); non-supplemented diet; non RF-exposed] (Tomruk, Guler & Dincel, 2010; Fletcher *et al.*, 2012; Yang *et al.*, 2013). Similarly, one study included data for a line of animals selected for high metabolic rate; we used data for the non-selected (control) line (Oldakowski *et al.*, 2012). For one other study, data were available for different age classes; we used data for individuals at peak fecundity (da Silva *et al.*, 2013).

We excluded certain types of studies in order to avoid sources of bias that could confound the meta-analyses. These included studies of *Bos taurus* (dairy cows); such animals have been selected for extreme reproductive output, and these studies invariably fail to include

an appropriate control (non-breeding) comparator (Metcalf & Monaghan, 2013). Two other studies were excluded because the effect of reproduction on oxidative damage could not be separated from a potential influence of environment (wild breeders compared with captive non-breeders) (Casagrande *et al.*, 2011), or because data were pooled for both sexes (Beaulieu *et al.*, 2011). Finally, we excluded one study of *Rattus norvegicus* (Sprague Dawley rats), because null results were mentioned in the text but details were not provided, and therefore effect sizes could not be calculated for these variables (Sainz *et al.*, 2000).

(2) Data extraction and calculation of effect sizes

Data for the analysis of oxidative damage in relation to reproductive effort were extracted from the text and tables in the form of Pearson's correlation coefficients, or slope estimates (and standard errors) from general linear models, sample sizes, and *P*-values. We also used dichotomous data in the form of means (\pm S.D.) for the comparison of individuals expending relatively high *versus* lower reproductive effort. Effect sizes for this diverse data set were calculated as described below. Similarly, data for the analysis of oxidative damage in breeders compared with non-breeders were extracted from the text and tables in the form of means (\pm S.D.) and sample sizes, or from graphs using GSYS version 2.4 graph digitising software (Nuclear Reaction Data Centre, Hokkaido University; www.jcprg.org/gsys/). For the following papers we requested and received additional information from the authors: Alonso-Alvarez *et al.* (2010), Bergeron *et al.* (2011), da Silva *et al.* (2013), Garratt *et al.* (2013), King, Garratt & Brooks (2013), Markó *et al.* (2011), Nussey *et al.* (2009), Xu *et al.* (2014). One study presented data in the form of medians, ranges and sample sizes (da Silva *et al.*, 2013); we estimated means (\pm S.D.) using standard formulae (Hozo, Djulbegovic & Hozo, 2005).

Statistics were converted to effect sizes in the form of Hedges' *g*, using Comprehensive Meta-Analysis (CMA) software (Borenstein *et al.*, 2005). Hedges' *g* is a measure of bias-corrected standardised mean difference which can be generated from categorical or continuous data (Hedges, 1981), and is therefore appropriate for diverse data sets. The data set used for analysis of oxidative damage in relation to reproductive effort included both continuous and dichotomous data as described above. Using Hedges' *g* as a standard measure of effect size allowed us to compare both qualitatively and quantitatively the differences in oxidative damage linked to breeding *per se*, and linked to relatively high *versus* lower reproductive effort. To infer the relationship between reproductive effort and oxidative damage on continuous scales, and to verify that results of the meta-analysis for reproductive effort were not biased by the method used for calculation of effect sizes, we also calculated Fisher's *Z* for all data points in reproductive

effort analyses. Fisher's *r*-to-*Z* transformation provides a variance-adjusted effect size, typically used to describe continuous data.

(3) Procedures for meta-analysis

Data were analysed in CMA (Borenstein *et al.*, 2005). Random-effects meta-analysis models were used, which weight data points according to a study sampling variance that is calculated based on the assumption that within-study sampling variance and between-study sampling variance are independent of one another (Borenstein *et al.*, 2010). This assumption is more accurate and more conservative compared to fixed-effects meta-analysis models, which assume that within- and between-study variances are equal, when analysing multiple data sets from different species. A tiered approach to analysis was taken: first, all tissues and all markers were analysed simultaneously. If this analysis showed $P \geq 0.05$ for the summary effect size, each tissue was then analysed independently to ascertain whether there were significant consistencies across markers within tissues. If this showed no significance ($P \geq 0.05$ for the summary effect size), then subsets of data for each individual tissue-marker combination were analysed, to ascertain whether specific markers within specific tissues showed significant consistencies in relation to reproduction. In 'all-tissues-all-markers' models, and 'individual-tissues-all-markers' models, where a study included data for multiple tissues and/or markers, the combined effect size was used. Similarly, where a study had reported both data from observational analyses and from experimental manipulation, the combined effect size was used.

Shared evolutionary history can potentially bias the results of meta-analyses (Chamberlain *et al.*, 2012). CMA is unable to scale models according to relatedness metrics, so in order to test the importance of phylogenetic influence we re-ran the analyses carried out in CMA, utilising MCMCglmm (Hadfield, 2010) in the R programming language (R Development Core Team, 2014). None of these models indicated an important phylogenetic component (see online File S1, Appendix S1). A phylogeny should not be included in an analysis when there is little or no support for a phylogenetic effect, as forcing a relatedness-based variance-covariance matrix in the random effects where there is no evidence to support the existence of an important influence of phylogenetic relatedness has the consequence of increasing credible intervals on fixed-effects estimates, and therefore increasing the likelihood of type II error. Therefore, results presented in the main text are derived from CMA.

(4) Evaluating potential sources of bias in the meta-analysis

Publication bias, i.e. the selective reporting of statistically significant results, can be a problem for meta-analyses (Borenstein *et al.*, 2005; Sterne, Becker & Egger, 2005; Koricheva *et al.*, 2014). We tested for evidence of publication bias using two methods: funnel plot, and trim-and-fill. Funnel plots represent a measure of study size (e.g. standard error) on the y-axis as a function of effect size on the x-axis; clustering of studies on one side of the mean or the other indicates bias, reflecting the fact that smaller studies (that are plotted towards the bottom) are more likely to be published if they have above-average effect sizes. Trim-and-fill is a method that trims any asymmetric studies from a funnel plot in order to calculate the unbiased effect size, and then fills the plot by reinserting the trimmed studies in addition to imputed counterparts to attain symmetry. Funnel plots are unlikely to be useful where there are fewer than $N=5$ studies in a meta-analysis (Sterne *et al.*, 2005), and therefore we used funnel plots and trim-and-fill in all instances where there were five or more studies. Results of these analyses are shown in File S1, Appendix S2.

Where existing studies have failed to detect an oxidative cost of reproduction, this might be because they have focussed on laboratory-housed animals rather than individuals living in natural populations. In addition, individuals that reproduce are unlikely to be a random subset of a population; breeders may be younger, higher quality or in better physiological condition than non-breeders. Therefore, studies might have failed to detect an oxidative cost of reproduction if they have relied on observational evidence, rather than having drawn animals from the same populations or social groups and randomly allocated them to breeding and non-breeding conditions (Selman *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014). Also, individuals may modulate their reproductive effort as a function of individual quality or condition; experimental manipulation of reproductive effort may therefore be necessary to detect an oxidative cost of reproduction (van Noordwijk & De Jong, 1986; Metcalfe & Monaghan, 2013). We evaluated whether there was any such evidence of systematic bias, by comparing the results of the full meta-analysis with results of meta-analyses that excluded different subsets of data in turn (laboratory studies; field studies; observational studies; studies that had not manipulated reproductive effort) (see online File S1, Appendix S2).

Heterogeneity analysis is a means of assessing the consistency in effect sizes across studies; significant heterogeneity may arise for example where there are interspecific differences in effect sizes that reflect the biology of species, or where there are methodological differences amongst studies (Koricheva *et al.*, 2014). Heterogeneity analyses were carried out using CMA

software, in terms of Cochran's Q which is calculated by summing the squared deviations of each study's estimate from the overall meta-analytic estimate, weighting each study's contribution as in the meta-analysis. P values are calculated using a χ^2 distribution with $k-1$ degrees of freedom (where k is the number of studies). In addition, we calculated I^2 [$100\% \times (Q - \text{d.f.})/Q$] which describes the proportion of total variation in studies estimates that is due to heterogeneity (Higgins & Thompson, 2002).

IV. RESULTS

(1) Reproductive effort and oxidative damage

Available data for the association between reproductive effort and oxidative damage came from eight studies of five species of mammals, and from three studies of three species of birds (Nussey *et al.*, 2009; Alonso-Alvarez *et al.*, 2010; Bergeron *et al.*, 2011; Garratt *et al.*, 2011, 2013; Markó *et al.*, 2011; Stier *et al.*, 2012; King *et al.*, 2013; Yang *et al.*, 2013; Xu *et al.*, 2014; Costantini *et al.*, 2014a) (see online File S1, Table S1). Consistent with the predicted



Fig. 1. Relationship between reproductive effort and oxidative damage. For studies that measured multiple tissues and/or markers, the combined effect size was used for analysis; see Sections III.2 and III.3 for details. Round symbols are effect sizes (Hedges' $g \pm 95\%$ confidence intervals) based on continuous data or categorical comparison of relatively high *versus* lower effort for the relationship between offspring number and oxidative damage in mothers. Open symbols show effect sizes for the individual studies; the black symbol is the summary (i.e. overall) effect size. There was a significant positive association between reproductive effort and oxidative damage (random effects meta-analysis: $Z = 2.714$, $N_{\text{studies}} = 11$, $P = 0.007$).

pattern, across all tissues and markers there was a significantly higher level of oxidative damage in mothers with relatively high compared with lower reproductive effort (Fig. 1). This result was qualitatively the same when effect sizes were calculated using Fisher's Z (random effects meta-analysis: Fisher's $Z = 0.176$; 95% confidence interval: lower limit, 0.047, upper limit, 0.305; $Z = 2.667$, $P = 0.008$). As Fisher's Z is a variance-adjusted form of a correlation coefficient, we can conclude from this that there is a continuously increasing relationship between offspring number and oxidative damage from relatively low to higher reproductive effort.

Markers of oxidative damage in blood plasma, serum, or erythrocytes were measured by nine of the studies included in the analysis, while four studies had measured such markers in organs (see online File S1, Table S1). We therefore examined whether the association between reproductive effort and oxidative damage held when considering blood and organ markers independently. Across all blood markers there was a significant positive association between reproductive effort and oxidative damage (Hedges' g : 0.341; 95% confidence interval: lower limit, 0.043, upper limit, 0.639; $Z = 2.245$, $P = 0.025$), whereas across all organ markers while the sign of the effect size was positive, no significant pattern existed (Hedges' g : 0.231; 95% confidence interval: lower limit, -0.242 , upper limit, 0.704; $Z = 0.957$, $P = 0.338$). Therefore, the positive association between reproductive effort and oxidative damage was largely driven by variation in levels of blood markers.

(2) Reproductive state and oxidative damage

To test the hypothesis that reproducing individuals have increased levels of oxidative damage, we next made categorical comparisons of breeders *versus* animals drawn from the same populations or social groups which had not bred. Available data for this comparison came from 15 studies of 11 species of mammals, and from 3 studies of 3 species of birds (Cederberg, Basu & Eriksson, 2001; Upreti *et al.*, 2002; Tomruk *et al.*, 2010; Bergeron *et al.*, 2011; van de Crommenacker *et al.*, 2011; Garratt *et al.*, 2011, 2013; Fletcher *et al.*, 2012; Oldakowski *et al.*, 2012; Stier *et al.*, 2012; da Silva *et al.*, 2013; Yang *et al.*, 2013; Cram *et al.*, 2015; Jan *et al.*, 2014; Schmidt, Blount & Bennett, 2014; Xu *et al.*, 2014; Costantini *et al.*, 2014b), and additional unpublished data from a study of banded mongooses (*Mungos mungo*) (see online File S1, Table S2). In a meta-analysis including all tissues and markers, there was no consistent association between reproduction and oxidative damage (Fig. 2). We therefore examined whether there was consistency across all markers of oxidative damage for each individual tissue type, for those tissues where multiple markers were available. Again, we found no consistent patterns for any tissue (Fig. 2). This was because, opposite to the predicted relationship, levels of malondialdehyde and protein carbonyls in heart were significantly lower

in breeders compared with non-breeders. Oxidised glutathione and protein thiols in heart did not vary consistently with reproductive state (Fig. 2). Similarly, levels of protein carbonyls in ovarian follicles were markedly and significantly lower in breeders compared with non-breeders (Fig. 2). In liver, malondialdehyde and oxidised glutathione showed considerable variation amongst studies and no consistent patterns, whereas protein carbonyls and protein thiols indicated markedly and significantly decreased levels of oxidative damage in reproductive compared to non-reproductive animals (Fig. 2). Skeletal muscle and kidney showed no consistent patterns for any marker of oxidative damage in relation to reproduction (Fig. 2).

In contrast to data for organs, blood markers of oxidative damage were borderline significantly higher in breeders compared with non-breeders in an analysis across all markers. This was because blood levels of protein carbonyls were markedly and significantly higher in breeders compared with non-breeders. However, levels of malondialdehyde, reactive oxygen metabolites and protein thiols showed no consistent patterns in relation to reproduction (Fig. 2).

There was no evidence to suggest that these results are confounded by publication bias, or by systematic bias due to data that were derived from laboratory studies, or field studies, or observational evidence (i.e. where animals had 'self-selected' whether to breed), or experiments that had not manipulated reproductive effort, respectively (see online File S1, Appendix S2). Surprisingly, however, the conclusion that reproductive effort is positively associated with oxidative damage was not changed in an analysis excluding data for manipulated reproductive effort (Hedges' g : 0.361; 95% confidence interval: 0.047 and 0.674; $Z = 2.254$, $N_{\text{studies}} = 9$, $P = 0.024$). By contrast, excluding all correlational data, i.e. data for breeders that had not experienced a manipulation of reproductive effort, rendered the relationship between reproductive effort and oxidative damage non-significant (Hedges' g : 0.123; 95% confidence interval: -0.262 and 0.509; $Z = 0.627$, $N_{\text{studies}} = 4$, $P = 0.531$; see online File S1, Table S4).

There was significant heterogeneity in effect size for several of the meta-analyses comparing breeders and non-breeders (Table 1).

V. DISCUSSION

Consistent with theory, using meta-analysis we found a significant positive association between reproductive effort and oxidative damage across tissues and markers. However, opposite to the pattern predicted based on existing theory, categorical comparisons of breeders *versus* non-breeders revealed that reproduction is associated with significant reductions in certain markers of oxidative damage in heart, liver and ovarian

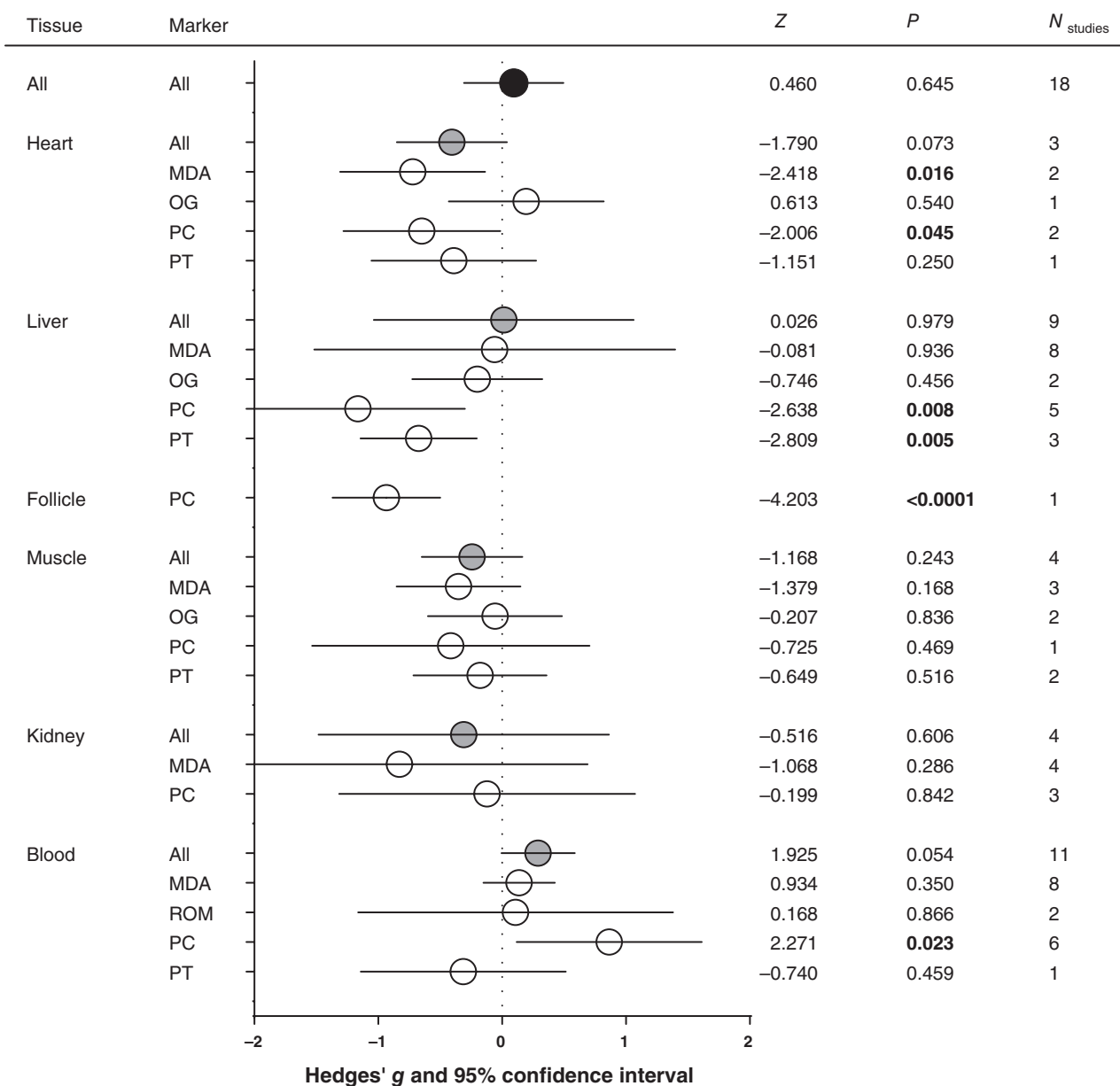


Fig. 2. Relationship between reproductive state and oxidative damage. Round symbols are effect sizes (Hedges' $g \pm 95\%$ confidence intervals) based on the difference in mean oxidative damage of breeders and non-breeders. The black symbol denotes an analysis of all-tissues-all-markers; the grey shaded symbols denote analyses of subsets of data for individual-tissues-all-markers; and the open symbols denote analyses of subsets of data for individual-tissues-individual-markers. Compared with non-breeders, negative values indicate lower oxidative damage in breeders, while positive values indicate higher oxidative damage in breeders. Markers are: MDA, malondialdehyde; OG, oxidised glutathione; PC, protein carbonyls; PT, protein thiols; ROM, reactive oxygen metabolites. The sign of the data for PT is reversed so negative values indicate decreased damage. Blood markers are plasma or serum. Z values and two-tailed P-values arising from random-effects meta-analysis models are shown; statistically significant effect sizes are shown in bold type. N_{studies} refers to the sample size of studies in each meta-analysis. See Sections III.2 and III.3 for details.

follicles. Figure 3 summarises these main findings of the meta-analysis schematically. Patterns of oxidative damage in other tissues showed no statistically significant associations with reproduction (Fig. 2). Blood protein carbonyls showed a contrasting pattern, levels being significantly increased in breeders compared with

non-breeders, while other blood markers did not differ significantly in association with reproductive state. Sample sizes were small for many of these comparisons, meaning that statistical non-significance can in many cases be attributed to a lack of power. Indeed, using Cohen's benchmarks for interpreting effect sizes, i.e.

Table 1. Heterogeneity analyses arising from the meta-analyses examining associations between reproduction and oxidative damage. Significant P values are shown in bold.

Comparison	Level of analysis		Q	d.f.(Q)	P	I ²
	Tissue	Marker				
Reproductive effort	All	All	13.971	10	0.174	28.425
Reproducing <i>versus</i> non-reproducing	All	All	90.435	17	<0.0001	81.202
	Heart	All	1.791	2	0.408	0.000
		MDA	0.125	1	0.724	0.000
		PC	0.116	1	0.734	0.000
	Liver	All	79.338	8	<0.0001	89.917
		MDA	103.159	7	<0.0001	93.214
		OG	0.671	1	0.413	0.000
		PC	17.049	4	0.002	76.539
		PT	0.442	2	0.802	0.000
		OG	0.020	1	0.887	0.000
	Muscle	All	0.221	3	0.974	0.000
		MDA	0.333	2	0.847	0.000
		OG	0.020	1	0.887	0.000
		PT	0.000	1	0.991	0.000
	Kidney	All	19.220	3	<0.0001	84.391
		MDA	31.363	3	<0.0001	90.435
		PC	11.198	2	0.004	82.140
	Blood	All	20.087	10	0.028	50.216
		MDA	11.027	7	0.137	36.522
		ROM	5.019	1	0.025	80.077
PC		25.216	5	<0.0001	80.171	

Heterogeneity analyses were carried out where $N_{\text{studies}} > 1$ (see Fig. 2). Markers are: MDA, malondialdehyde; PC, protein carbonyls; OG, oxidised glutathione; PT, protein thiols; ROM, reactive oxygen metabolites.

Hedges' g of 0.2, 0.5, and 0.8, as small, medium, and large, respectively (Cohen, 1988), and considering data for all organ tissues and individual markers, we find that there are eight large, three medium and two small effect sizes, while only two effect sizes can be considered trivial. All but one of these effect sizes are negatively signed. Considering data for individual markers in blood, we find that there are two large positively signed effect sizes, one medium negatively signed effect size, and one trivial (positively signed) effect size.

The reduction in oxidative damage in breeders is not simply because reproducing animals were in better physiological condition or were younger; the same pattern was observed when including only data from experiments that randomly allocated individuals to treatment groups (see online File S1, Appendix S2). Such experiments typically confirmed that females allocated to breeding and non-breeding groups were of similar body mass and age (Tomruk *et al.*, 2010; Garratt *et al.*, 2011, 2013; Stier *et al.*, 2012; da Silva *et al.*, 2013; King *et al.*, 2013; Pichaud *et al.*, 2013; Yang *et al.*, 2013), and therefore confounding biases in oxidative damage would not be expected. These consistent but contrasting relationships between oxidative damage and reproductive effort, *versus* the reproductive state *per se*, may help explain why many existing studies have concluded that reproduction has little or no oxidative cost. Rather, we hypothesise that the cost of reproduction could be mediated by dual impacts of oxidative damage on mothers

and offspring, while mothers may face additional costs diminishing such damage.

Since our meta-analysis was based on data for multiple tissues and markers of oxidative damage, and several different species of mammals and birds, heterogeneity in effect sizes is to be expected. Values for I^2 of 25, 50, and 75%, represent low, medium, and high levels of heterogeneity, respectively (Higgins *et al.*, 2003). Indeed, values for Cochran's Q and I^2 show that there is significant and marked heterogeneity in effect sizes amongst studies for some tissue–marker combinations (Table 1). Such heterogeneity is likely to arise because of variation in the biology of different species, but also methodological differences amongst studies (e.g. Costantini & Møller, 2008). For example, most studies in the meta-analyses that reported malondialdehyde as a marker of oxidative damage used a spectrophotometric assay that has been criticised for lack of specificity (reviewed by Monaghan *et al.*, 2009; Selman *et al.*, 2012), while a few recent studies have used a more precise HPLC method (e.g. Cram *et al.*, 2015; Schmidt *et al.*, 2014). Across all tissues and all markers, effect sizes were fairly consistent ($I^2 = 28\%$) for the relationship between reproductive effort and oxidative damage, while having a high level of heterogeneity ($I^2 = 81\%$) for the association between reproductive state and oxidative damage. The reason for this difference is unclear, but it may simply be because most data relating to reproductive effort were for blood markers, whereas

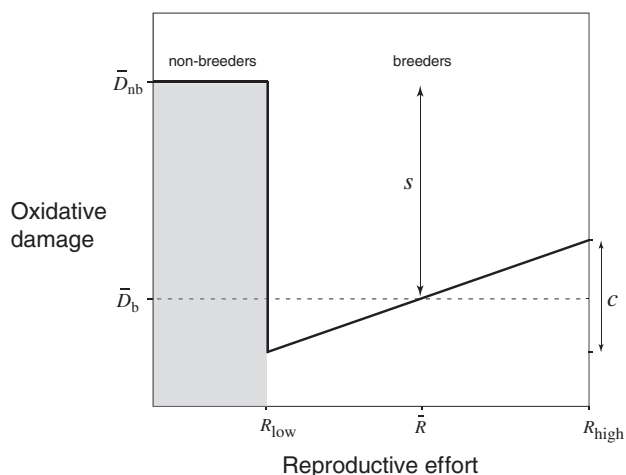


Fig. 3. Hypothesised schematic relationship between reproduction and oxidative damage, based on the results of the meta-analyses. The quantity s ('shielding') is the Hedges' g effect size for the difference in mean oxidative damage between non-breeders (\bar{D}_{nb}) and breeders (\bar{D}_b). From our analysis the value of s is 0.828 (mean of point estimates for malondialdehyde in heart; protein carbonyls in heart, liver, and follicles; and protein thiols in liver; Fig. 2). Oxidative damage correlates positively with reproductive effort (based on continuous data and categorical comparison of relatively high *versus* lower effort; Fig. 1). We can represent the magnitude of this increase as Hedges' g by dichotomising continuous data into 'high' (R_{high}) and 'low' (R_{low}) categories, above or below the mean reproductive effort of all breeders, \bar{R} , respectively. The magnitude of this effect (denoted c , 'cost of reproduction') is 0.343. The range R_{low} to R_{high} is standardised as length 1; lines s and c are drawn in proportion to this unit length.

data relating to reproductive state were for a wide variety of tissues. The existence of significant heterogeneity highlights the importance of using random-effects meta-analyses when analysing multiple data sets from different species. Random-effects models are relatively conservative because they do not assume homogeneity in effect size across studies, an assumption that is unlikely to hold when comparing results from multiple species or methodological approaches (Borenstein *et al.*, 2010).

(1) Why is oxidative damage reduced in reproducing females?

We hypothesise that transition to the reproductive state triggers a pre-emptive reduction in levels of oxidative damage in certain markers and tissues, in order to shield mothers, and in particular their gametes and developing offspring, from harm caused by an inevitable increase in oxidative damage resulting from expenditure of reproductive effort. If, as we hypothesise, such pre-emptive reductions in oxidative damage serve to shield mothers and offspring from oxidative insults during sensitive

periods of reproduction, the timing of such 'oxidative shielding' is likely to differ amongst taxa. We now discuss the hypothesised importance of oxidative shielding in relation to maternal life histories, follicular growth, placental function and lactation in eutherian mammals, and in relation to egg production in oviparous species.

(a) Oxidative shielding in eutherian mammals

Reproductive effort is positively correlated with oxidative damage (Fig. 1). Mothers might therefore gain from pre-emptive reduction of oxidative damage in preparedness for reproduction, to reduce the oxidative costs of a given level of reproductive effort, or to ensure that levels of oxidative damage do not subsequently exceed some critical threshold above which it may be impossible to maintain cellular homeostasis. For example, uncontrolled oxidative stress arising from reproductive effort could result in the proteolytic capacity of cells becoming overwhelmed by large numbers of oxidized protein molecules, leading to impaired proteolytic digestion. Rather than being broken down, such oxidized proteins may instead become aggregated, and impair cell and organ function (Davies, 2000).

We hypothesise that reductions in oxidative damage in reproducing females may also serve to shield gametes and developing offspring. In placental mammals, oxygen tension is an important regulator of trophoblast cell proliferation and differentiation (Bose *et al.*, 2006). During early pregnancy, ROS regulate various cell functions and physiological hypoxia of the gestational sac is thought to protect the foetus from oxidative damage (Bose *et al.*, 2006; Jauniaux, Poston & Burton, 2006). This is supported by animal studies which have demonstrated increased blastocyst developmental rate at lower oxygen tension (Gupta *et al.*, 2007). However, at the onset of embryonic aerobic metabolism, induced by the establishment of utero-placental blood flow, oxygen tensions inside the intervillous space increase leading to a burst of oxidative stress (Gupta *et al.*, 2007; Al-Gubory, Fowler & Garrel, 2010). High levels of placental and systemic oxidative stress in mothers have been linked to altered placental vasculature, defective embryogenesis and pregnancy loss (Gupta *et al.*, 2007; Al-Gubory *et al.*, 2010). One mechanism linked to pregnancy loss in humans is antiphospholipid (aPL) antibody syndrome: oxidative damage to low density lipoproteins (LDLs) can alter the antigenic properties of modified phospholipids resulting in formation of aPL antibodies directed against them (Gupta *et al.*, 2007; Vial *et al.*, 2013). In laboratory rodents, up to 10% of pregnancies fail post-implantation (Wilmot, Sales & Ashworth, 1986). Comparable data are limited for other taxa, and non-existent for natural populations of animals (Jauniaux *et al.*, 2006). In order to assess the potential adaptive significance of oxidative shielding, it would be valuable to gather more data on associations between oxidative

stress and pregnancy outcomes in a broader range of species, including studies in the wild.

Sub-lethal effects of placental oxidative stress on embryos are also likely to be very important, and have been highlighted as a potential general mechanism linking altered placental function to life-history trajectories, i.e. 'fetal programming' (Myatt, 2006). For example, placental dysfunction caused by oxidative stress has been linked to fetal growth restriction and low birth weight in humans (Gupta *et al.*, 2007; Al-Gubory *et al.*, 2010). Studies of natural populations of mammals have shown that conditions experienced in natal environments can impact on reproduction and survival in later life (Nussey *et al.*, 2007; Bouwhuis *et al.*, 2010). Oxidative stress is a candidate mechanism underlying such effects (Isaksson *et al.*, 2011), but we are not aware of any studies that have examined links amongst oxidative stress, placental dysfunction and life-history trajectories in any non-human animal species. This seems likely to be a worthwhile direction for future research.

Lactation imposes huge nutritional demands on mothers: organs such as the liver and alimentary tract grow, while stored reserves are catabolised (Hammond *et al.*, 1994; Speakman, 2008) which increases ROS production and the risk of oxidative stress (Schaff *et al.*, 2012). Oxidative stress in lactating females can have several deleterious impacts on offspring, for example by reducing milk yield (Suriyasathaporn *et al.*, 2009; Chandra *et al.*, 2013), and by impairing colostrum and milk quality in terms of antioxidant content (Rizzo *et al.*, 2013a). At parturition offspring are at risk of oxidative stress as they are exposed to atmospheric concentrations of oxygen; antioxidant enzymes and vitamins in colostrum and milk therefore confer protection against oxidative stress during neonatal development (Castillo *et al.*, 2005; He *et al.*, 2008; Albera & Kankofer, 2009). Finally, malondialdehyde (MDA) is present in milk (Wicheansoni *et al.*, 2007; Suriyasathaporn *et al.*, 2009; Rizzo *et al.*, 2013b), and levels of MDA in maternal blood and milk are correlated in dairy cows (Bouwstra *et al.*, 2008). MDA is highly reactive and may therefore initiate lipid peroxidation in milk and reduce its nutritional quality. Furthermore, ingestion of oxidised fats and MDA in milk could cause lipid peroxidation *in vivo* and be detrimental to offspring health (Serini *et al.*, 2011). We therefore hypothesise that mothers are selected to shield offspring from such effects by reducing levels of oxidative damage during lactation.

The majority of studies of mammals used in the meta-analysis comparing breeders and non-breeders presented data for lactating females, whereas three studies presented data for females during pregnancy, and one study presented data for ovarian follicles (see online File S1, Table S2). We hypothesise that levels of oxidative damage will be consistently reduced from oocyte maturation through gestation and lactation in mammals, compared to non-breeding periods. However, to

the best of our knowledge, no studies to date have measured oxidative damage levels in the same individuals during pre-breeding, pregnancy, lactation, and again post-breeding for any animal species. Collection of these kinds of longitudinal data would be extremely valuable to ascertain whether reductions in oxidative damage are of similar or different magnitude across these distinct stages of reproduction.

(b) Oxidative shielding in oviparous animals

Our meta-analysis comparing breeders and non-breeders also included data for egg-laying females of three species of birds (see online File S1, Table S2). In oviparous species, the egg provides all the nutrients required for embryonic development; oxidative stress could be deleterious for the production and viability of eggs if it resulted in depletion of lipids and proteins, or antioxidants (carotenoids, and vitamins A and E) that are required for albumen and yolk synthesis (Blount, Houston & Møller, 2000). In addition, oxidative stress in females during egg production has the potential to result in damage to specific proteins, such as immunoglobulins that are required to confer passive immunity for offspring (Haq, Bailey & Chinnah, 1996), and lysozymes that confer anti-bacterial defence in eggs. Furthermore, aldehydic lipid peroxidation products such as malondialdehyde are incorporated into the lipid matrix of yolk (Mourente *et al.*, 1999; Mohiti-Asli *et al.*, 2008), which may cause oxidative degeneration of yolk nutrients (Grune *et al.*, 2001), and lipid peroxidation *in vivo*. Studies of natural populations of birds have shown that females with greater resistance to oxidative stress produce larger clutches with higher hatching success (Bize *et al.*, 2008), and have linked conditions experienced in the natal environment to adult reproduction and senescence (Millon *et al.*, 2011; Cartwright *et al.*, 2014). It would therefore be interesting to examine how maternally-derived oxidative damage in eggs may affect offspring. To date, no study has examined whether levels of oxidative damage in mothers and their eggs and offspring are correlated, or how maternally-derived oxidative damage may influence the life histories of offspring.

In oviparous animals which reproduce only once in a single season, we hypothesise that levels of oxidative damage will be consistently reduced in mothers during conception and egg production, compared to incubation, provisioning or non-breeding periods. For species that lay multiple times over a single breeding season, it would be interesting to examine whether any decrease in levels of oxidative damage is maintained throughout all phases of reproduction, or alternatively, whether oxidative damage is diminished only during conception and egg production. The latter may be expected, because oxidative shielding presumably incurs costs (otherwise females would maintain low levels of oxidative damage all of the time). Finally, for oviparous mammals (i.e. monotremes) that lactate, or birds

that provision their young with crop secretions (e.g. columbids), it would be interesting to investigate whether oxidative damage products are found in these fluids, and if so, whether levels of oxidative damage are decreased in mothers while they provision dependent offspring.

(2) Consistency and inconsistency amongst markers of oxidative damage

It has previously been emphasised that levels of oxidative damage may vary amongst specific markers and tissues, underlining the importance of measuring multiple markers and (if possible) different tissues when studying oxidative stress (reviewed in Costantini, 2008; Selman *et al.*, 2012). The meta-analysis results support this view, in so far as categorical comparisons of breeders and non-breeders revealed tissue- and marker-specific associations between reproduction and oxidative damage. In breeders, oxidative damage was significantly reduced in liver and heart – metabolically active organs that increase in mass during lactation, presumably to optimise processing of nutrients and elimination of waste products (Hammond *et al.*, 1994). Levels of oxidative damage were also significantly decreased in ovarian follicles from cyclic compared with acyclic water buffalos (*Bubalus bubalis*) (Jan *et al.*, 2014). ROS have important signal functions during follicular development, but are also responsible for initiation of apoptosis in primordial, primary and antral follicles, whereas antioxidants protect against oxidative-damage-induced follicular apoptosis (Gupta *et al.*, 2007; Jan *et al.*, 2014). In addition, oxidative damage to germline DNA could result in genetic changes being transmitted to offspring *via* common genetic inheritance (mutations) and/or epigenetic inheritance, with deleterious consequences for offspring fitness (Velando, Torres & Alonso-Alvarez, 2008). Oxidative shielding in these organs is therefore likely to have an important role in maintaining functions which are vital for successful reproduction and the production of high-quality offspring.

The significant positive association between reproductive effort and oxidative damage was largely driven by data for various blood markers (Fig. 1), while in stark contrast to data for organ markers, categorical comparison revealed that blood protein carbonyls were significantly higher in reproductive compared with non-reproductive females (Fig. 2). This suggests that blood markers provide a signature of current reproductive effort, which organ markers do not provide over equivalent, short timescales. It would therefore be interesting to assess whether blood markers of oxidative damage are decreased upon transition to the reproduction state, in line with patterns observed for organs.

Why blood protein carbonyls in particular are significantly increased in breeders is unclear. One possibility is that increased protein turnover in reproducing animals results in greater availability of substrates that are

susceptible to carbonylation. Alternatively, carbonylation may serve an important ‘protein quality control’ function, signalling mistranslated or otherwise aberrant proteins for proteolytic degradation to prevent their incorporation into biomolecules involved in information transfer (e.g. ribosomes; RNA and DNA polymerases), while also yielding amino acids for *de novo* protein synthesis (Nystrom, 2005). To help separate these possibilities, it would be interesting to examine whether blood levels of protein carbonyls correlate positively or negatively with reproductive success. Ultimately, to improve our understanding of the role played by oxidative stress in life-history evolution, we need more studies that link variation in individual markers of oxidative damage to functional outcomes, particularly reproductive success and survival (Selman *et al.*, 2012; Metcalfe & Monaghan, 2013). Such evidence is still remarkably scarce (see for example Noguera, Kim & Velando, 2012).

(3) How is oxidative damage reduced in reproducing females?

Oxidative damage could be decreased by various potential mechanisms. An obvious possibility is *via* increased levels of antioxidant defences. Consistent with this explanation, studies have linked decreased levels of lipid and protein oxidation to increased total glutathione (tGSH) and total superoxide dismutase (SOD) activity in the liver of reproducing wild-derived house mice (*Mus musculus domesticus*) (Garratt *et al.*, 2011, 2013), increased liver SOD and non-enzymatic antioxidant activity (total antioxidant capacity, TAC) in lactating Mongolian gerbils (*Meriones unguiculatus*) (Yang *et al.*, 2013), and increased liver SOD activity in lactating Brandt’s voles (*Lasiopodomys brandtii*) (Xu *et al.*, 2014). Such increased activity of antioxidant defences could be triggered by changes at the cellular, mitochondrial level (Pichaud *et al.*, 2013). Lactating mice have lower electron transport system activity and higher mitochondrial density in liver compared with non-breeders, which could in turn lead to upregulated antioxidant defences (Pichaud *et al.*, 2013).

A review of the broader literature including studies of birds reveals that relationships between reproduction and antioxidant defences and/or resistance to oxidative stress are often contradictory (Stier *et al.*, 2012). Indeed, several studies of birds have reported that blood levels of antioxidant defences are decreased during reproduction (Alonso-Alvarez *et al.*, 2004; Wiersma *et al.*, 2004; Losdat *et al.*, 2011). This apparent discrepancy among taxa might be because existing studies of mammals have measured antioxidant defences in various tissues, sometimes including blood, whereas studies of birds have invariably measured antioxidant defences in blood only. Across taxa, there is a tendency for increased blood levels of oxidative damage in breeders (Fig. 2). In breeding birds, it has been argued that reduced blood

levels of antioxidant defence represent a cost of reproduction, i.e. the defences are sacrificed (Alonso-Alvarez *et al.*, 2004; Wiersma *et al.*, 2004; Losdat *et al.*, 2011). Alternatively, it seems possible that such reductions in antioxidant defences might allow increased levels of oxidative damage to circulating macromolecules, specifically in order to yield e.g. amino acids for *de novo* synthesis of nutrients (discussed above; see Section V.2). Ultimately, to separate these possibilities we will need greater replication in measurements of specific markers of oxidative damage across tissues and taxa, coupled with studies of how variation in specific markers correlates with fitness.

A decrease in oxidative damage in breeders might also be explained by uncoupling proteins (UCPs), reducing ROS production in mitochondria (Brand, 2000). However, UCP gene expression is downregulated or unchanged during lactation (Speakman, 2008), making mitochondrial uncoupling an unlikely general explanation for decreased oxidative damage in breeders. Moreover, mitochondrial uncoupling seems an unlikely mechanism by which animals would decrease levels of oxidative damage during reproduction, because this would result in reduced efficiency of energy production at a time when metabolic demands are likely to be particularly high. Additional studies are required to assess whether uncoupling is associated with decreased oxidative damage during other stages of reproduction, in other tissues, and in other taxa. Decreased levels of oxidative damage in breeding females might further be explained by the action of oestradiol. Studies of rats have shown that ovariectomy leads to increased oxidative damage, whereas oestrogen administration increases levels of glutathione and glutathione peroxidase (GPx) activity, and results in decreased lipid peroxidation in various tissues (Persky *et al.*, 2000; Borrás *et al.*, 2003; Kireev *et al.*, 2007; Aksakal *et al.*, 2011). *In vitro* experiments have confirmed that oestradiol acts by binding to oestrogen receptors and subsequently activates the mitogen activated protein (MAP) kinase and nuclear factor kappa B (NF- κ B) signalling pathways, causing increased expression of genes that code for antioxidants such as SOD and GPx (Vina *et al.*, 2005).

We have hypothesised that reduced oxidative damage in certain tissues of reproducing females is an adaptive response to enhance reproductive success. However, we must consider the alternative possibility that a simple dilution effect might explain reduced levels of oxidative damage in tissues such as heart, liver and ovarian follicles, which become enlarged during reproduction. As previously argued, dilution seems an unlikely explanation for decreased oxidative damage in breeders, because: (i) not all markers of oxidative damage in such tissues are significantly lower in breeders compared with non-breeders, see Fig. 2; (ii) the fact that certain antioxidants have been measured at higher levels in such tissues in breeders is inconsistent

with a dilution effect (Garratt *et al.*, 2011, 2013; Yang *et al.*, 2013; Xu *et al.*, 2014); and (iii) blood volume also increases in lactating mammals, but there is little or no indication that blood markers of oxidative damage are reduced in breeders (Yang *et al.*, 2013; and see Fig. 2).

(4) How costly is the diminishment of oxidative damage?

To what extent diminishment of oxidative damage is costly for breeding females is an important question, because costs of reproduction are likely mediated both by oxidative damage and by mechanisms to minimise or repair such damage (Monaghan *et al.*, 2009; Selman *et al.*, 2012). Such costs may depend on the mechanism responsible for decreased oxidative damage. For example, since antioxidant defences have low energetic costs, it has been argued that nutrition *per se* is unlikely to limit protection against oxidative damage (Speakman & Garratt, 2014). However, the efficiency of energy production is decreased by mitochondrial uncoupling (Brand, 2000), which could hypothetically lead to energy limitation of foraging capacity, and therefore reduced ability to sequester resources required to initiate or sustain reproductive effort. Such resources could include macronutrients, but also micronutrients such as vitamins A, C and E, flavonoids, and carotenoids, which may themselves be limiting for reproduction and potentially also antioxidant defence (Monaghan *et al.*, 2009). Alternatively, at physiological levels ROS have important roles in various signalling transduction pathways (Al-Gubory *et al.*, 2010; Metcalfe & Alonso-Alvarez, 2010), and therefore if oxidative shielding reduces ROS production this could impact on diverse aspects of cell and organ function.

If reducing oxidative damage is costly, mothers might be selected to show graded rather than all-or-nothing reductions in oxidative damage on transition to the reproductive state, proportional to the scale of their reproductive investment (i.e. offspring number). Recent research on house mice has found significant differences in mitochondrial function and density in reproductive compared with non-reproductive females, while there were no such differences in relation to experimentally manipulated litter size (Pichaud *et al.*, 2013). This could indicate that physiological restructuring associated with reproduction, e.g. in mitochondria, represents an all-or-nothing transition, rather than varying in relation to reproductive effort (Pichaud *et al.*, 2013). However, experimental manipulations of offspring number are usually carried out post-birth, by which time mothers have already undergone extensive physiological restructuring, and further rapid modification may not be possible within the remaining period of parental care.

It has recently been argued that the ability to detect an oxidative cost of reproduction could critically depend

on experimental manipulation of brood or litter size, to overcome the potential problem that individuals may adjust their reproductive effort to their own quality or access to resources (Metcalf & Monaghan, 2013). However, we found no consistent evidence to suggest that the results of the meta-analysis were confounded by studies that have failed to manipulate reproductive effort. On the contrary, the conclusion that reproductive effort is positively associated with oxidative damage was not changed in an analysis based on correlational data only, i.e. excluding data for manipulated reproductive effort. By contrast, excluding data for breeders that had not experienced a manipulation of reproductive effort rendered this relationship non-significant (see online File S1, Table S4). We can suggest three, non-mutually exclusive possible explanations for this discrepancy between correlational and experimental data. Firstly, females may not be able to readjust their physiology rapidly to the altered workload imposed by experimental manipulation of offspring number when it is carried out post-birth, as discussed above. If reductions in oxidative damage in breeding females are graded, it seems possible that the degree of reduction is set when initial decisions about offspring number are made (i.e. at follicle enlargement and ovulation). To test this idea experimentally would require manipulation of follicle number, for example by surgical intervention (e.g. Sinervo *et al.*, 1990), or *via* egg removal and egg addition manipulations in oviparous species that are indeterminate layers.

Secondly, we must consider the possibility that individuals with relatively high baseline (i.e. pre-breeding) levels of oxidative damage subsequently produce more offspring. It is clear that metabolic rate often increases during the early stages of reproduction, for example due to costs of egg production in birds (e.g. Vézina & Williams, 2002). Such increased metabolic rate in early reproduction may be expected to incur an oxidative cost, and hence result in there being a positive correlation between reproductive effort and levels of oxidative damage when such measurements are made several days later during peak reproduction. We would interpret such an association as evidence to suggest that reproduction has an oxidative cost. However, we are not aware of any evidence which links high levels of oxidative damage prior to breeding and high subsequent levels of offspring production. On the other hand, two studies have reported that levels of oxidative damage in females before, or early during reproduction, are significantly negatively correlated with the number of offspring that are subsequently produced (Costantini, Carello & Fanfani, 2010; Stier *et al.*, 2012). This suggests that pre-breeding oxidative state may constrain reproduction (Dowling & Simmons, 2009; Stier *et al.*, 2012).

Thirdly, it is possible that a positive correlation between reproductive effort and oxidative damage

exists for breeders that had not experienced a manipulation of reproductive effort, as a consequence of 'individual quality' effects. For example, individuals that can afford high levels of reproductive effort may also be able to cope with high levels of oxidative damage, but this does not necessarily mean that increased reproductive effort causes increased oxidative damage. Similarly, the positive association between reproductive effort and oxidative damage could arise due to 'terminal investment', i.e. individuals in poorer physiological condition, with higher levels of oxidative damage and lower future survival prospects, may invest more in current reproduction. However, if this was true we may expect a non-linear association between reproduction and oxidative damage.

We conclude that since the positive correlation between reproductive effort and oxidative damage shown by our meta-analysis only holds for observational data, further experimental research will be needed to determine its causal basis. Nevertheless, whether or not reproduction causes oxidative damage, the idea that breeding females may be selected to reduce levels of oxidative damage in order to protect their developing offspring remains a valid hypothesis. Data from longitudinal sampling, and detailed cross-sectional sampling pre-, during and post-reproduction will be critical for our understanding of the temporal dynamics of changes in oxidative damage and the potential costs. Indeed, only longitudinal data can reveal whether pre-breeding oxidative state influences an individual's future reproductive effort (Stier *et al.*, 2012). A capacity to meet the costs of oxidative shielding might influence an individuals' reproductive effort, or whether they breed at all. To explore these possibilities we need studies of how pre-breeding oxidative state predicts subsequent reductions in oxidative damage associated with reproduction, and whether such changes correlate with variation in offspring number.

Finally, our meta-analysis considered only females; this reflects a bias in the published literature towards studies of females, presumably because it is often relatively difficult to measure reproductive effort in males (e.g. the cost of territory guarding and defence, and the cost of mate searching and guarding). Mitochondrial DNA appears to be particularly susceptible to oxidative damage, due to its proximity to the major source of ROS within cells (reviewed by Monaghan *et al.*, 2009). On the assumption that reducing oxidative damage incurs energetic or other costs (discussed above; Section V.4), it seems possible that females may pay a higher cost of reproduction than males due to oxidative shielding. If so, oxidative shielding might be a contributory factor underlying the evolution of inter-sexual conflict. However, several authors have emphasised the importance of oxidative damage as a mechanism responsible for decreased sperm viability and damage to male germline DNA; such changes in DNA

could be transmitted to offspring *via* common genetic inheritance (mutations) and/or epigenetic inheritance, with deleterious consequences for the fitness of offspring. Females should be selected to avoid mating with such males (Blount, Møller & Houston, 2001; Velando *et al.*, 2008; Dowling & Simmons, 2009; Metcalfe & Alonso-Alvarez, 2010). Males, which we have not considered in our meta-analysis, might therefore gain from oxidative shielding of sperm to increase mating success or the viability of resulting offspring. Indeed, oxidative shielding could have an important role in preventing oxidative damage to germline DNA transmitted *via* the maternal or paternal line. Additional studies of temporal patterns of oxidative damage in reproducing and non-reproducing males would therefore be valuable.

VI. CONCLUSIONS

(1) Our meta-analysis using data for females of homeothermic species reveals that, consistent with existing theory to explain the cost of reproduction, increased reproductive effort is associated with greater oxidative damage. This correlation holds for observational data, but not for data arising from experimental manipulations of reproductive effort. Therefore, further experimental research will be needed to determine its causal basis.

(2) Contrary to existing theory to explain the cost of reproduction, we found that transition to the reproductive state is associated with markedly reduced levels of oxidative damage in certain markers and tissues.

(3) We hypothesise that such reductions in oxidative damage are pre-emptive and function to shield mothers, and in particular their gametes and developing offspring, from critical levels of oxidative insults that inevitably increase as a consequence of reproductive effort. We refer to this idea as the ‘oxidative shielding hypothesis’.

(4) The oxidative shielding hypothesis could explain why many existing studies have concluded that reproduction has little or no oxidative cost. Rather, according to our analysis, the cost of reproduction seems likely to be mediated by dual impacts of oxidative damage on mothers and offspring, while mothers may face additional costs diminishing such damage.

(5) We suggest that a valuable direction for future studies of the cost of reproduction would be to quantify the magnitude of the different costs associated with sustaining oxidative damage in mothers and their offspring, and those borne by mothers in the reduction of oxidative damage.

(6) It would also be interesting to extend the range of studies that quantify the oxidative cost of reproduction, to include additional data for males, and to examine whether breeding males exhibit ‘oxidative shielding’

and in which tissues. Similarly, it would be valuable to gather data for oxidative damage in organs of birds in relation to reproductive effort and reproductive state.

(7) Current theory to explain the cost of reproduction focusses on the individual fitness costs faced by breeders. We suggest that future advance in life-history theory needs to take account of potential transgenerational impacts of the mechanisms underlying life-history trade-offs.

VII. ACKNOWLEDGEMENTS

We are very grateful to C. Alonso-Alvarez, M. S. Benfato, P. Bergeron, D. Costantini, M. Garratt, G. Markó, D. H. Nussey, C. Schmidt and Y.-C. Xu for providing further details on published analyses to enable their inclusion in the meta-analysis. We are also very grateful to M. J. P. Simons for discussion and advice regarding meta-analytical methods, and to N. B. Metcalfe, two anonymous reviewers, and the editors for many helpful suggestions which greatly improved the manuscript.

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IX. SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

File S1. Oxidative shielding and the cost of reproduction.

(Received 22 July 2014; revised 30 January 2015; accepted 12 February 2015)