TECHNICAL COMMENT

Measuring Oxidative Stress: The Confounding Effect of Lipid Concentration in Measures of Lipid Peroxidation

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ABSTRACT

Lipid peroxidation products are widely used as markers of oxidative damage in the organism. To properly interpret the information provided by these markers, it is necessary to know potential sources of bias and control confounding factors. Here, we investigated the relationship between two indicators of lipid mobilization (circulating levels of triglycerides and cholesterol) and two common markers of oxidative damage (plasma levels of malondialdehyde and hydroperoxides; the latter estimated from the d-ROMs assay kit). The following five avian species were studied: red-legged partridge (*Alectoris rufa*), zebra finch (*Taeniopygia guttata*), spotless starling (*Sturnus unicolor*), marsh harrier (*Circus aeroginosus*), and Montagu's harrier (*Circus pygargus*). In all cases, plasma triglyceride levels positively and significantly correlated with lipid peroxidation markers, explaining between 8% and 34% of their variability. Plasma cholesterol, in contrast, showed a significant positive relationship only among spotless starling nestlings and a marginally significant association in zebra finches. These results indicate that lipid peroxidation marker levels covary with circulating lipid levels. We discuss the potential causes and implications of this covariation and recommend that future studies that measure oxidative damage using lipid peroxidation markers report both raw and relative levels (i.e., corrected for circulating triglycerides). Whether the observed pattern also holds for other tissues and in other taxa would deserve further research.

Introduction

Oxidative stress is commonly defined as the imbalance between the levels of prooxidants and antioxidants in favor of the former, leading to oxidative damage (Halliwell and Gutteridge 2007). It has attracted considerable interest in the field of biomedicine during the past decades (Halliwell and Gutteridge 2007). Oxidative stress is involved, either as an ultimate cause or as a simple mediator, in the etiology of nearly 150 disorders, including several types of cancer, autoimmune disorders, and degenerative processes associated with aging (Halliwell and Gutteridge 2007). More recently, evolutionary biologists and ecologists have underlined the importance of oxidative stress as a key mediator in life-history trade-offs, probably playing a relevant role in development, reproduction (including honest sexual signaling), and senescence (von Schantz et al. 1999; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Costantini 2014).

However, measuring oxidative stress is not an easy task. On the one hand, direct analyses of reactive oxygen species in biological materials are difficult because of their intrinsic reactivity and short half-lives and because currently available methods have a limited applicability to studies in the wild. On the other hand, antioxidant defenses comprise a wide array of compounds of different chemical nature, which makes it unfeasible to simultaneously quantify most of them. More importantly, levels of antioxidants per se may not reflect the strength of antioxidant defenses. This is because in vivo antioxidant capacity is not a simple additive function of the concentration of individual antioxidants in a sample due to the

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synergistic and antagonistic interactions among them (Monaghan et al. 2009; Pérez-Rodríguez 2009). Also, levels of antioxidants often result from dynamic and adaptive responses to oxidative challenges (Costantini and Verhulst 2009; Pérez-Rodríguez 2009), making it difficult to assess the oxidative status of the individual by quantifying antioxidant defenses alone.

Given the limitations to infer oxidative stress from the two components of the oxidative equilibrium alone, the commonest approach is to quantify the end results of this (un)balance, using markers of oxidative damage (Halliwell and Gutteridge 2007). A range of techniques allows measuring oxidative damage to DNA, proteins, or lipids (Halliwell and Gutteridge 2007; Mateos and Bravo 2007). Measuring oxidative damage is frequently done by quantifying peroxidation products of lipids, usually polyunsaturated fatty acids, which are very susceptible to attack by free radicals (Mateos and Bravo 2007). Lipid peroxidation usually proceeds as a chain reaction leading to the production of several intermediate compounds, often referred to as reactive oxygen metabolites (ROMs). ROMs are mostly composed of lipid hydroperoxides, which have been extensively used as biomarkers of oxidative damage. With the recent popularization of commercial d-ROMs assay kits (Alberti et al. 2000), the use of ROMs has become much more frequent among ecophysiologists and evolutionary ecologists. In any case, lipid hydroperoxides are not endpoint products in the chain reaction, and they further decompose into other more stable substances such as aldehydes or isoprostanes (Halliwell and Gutteridge 2007). In fact, quantification of malondialdehyde (MDA) levels is the most common approach for the assessment of lipid peroxidation in biological and medical sciences (Halliwell and Gutteridge 2007; Mateos and Bravo 2007; Monaghan et al. 2009).

However, like other markers of oxidative damage, both ROMs and MDA levels have a number of limitations and sources of error, such as the contribution of metabolites derived from the diet or the potential interference of nontarget compounds in the analyses (Halliwell and Gutteridge 2007; Monaghan et al. 2009; Kilk et al. 2014). Another potential source of bias has been more rarely considered and is the focus of this work: the influence that the lipid content of the samples (blood, tissue) used for analysis may have on the measured levels of lipid peroxidation markers. If lipid peroxidation markers result from the action of ROMs on lipids, it can be expected that higher lipid content would result in an enhanced peroxidation cascade due to the greater abundance of peroxidizable substrates. This may ultimately bias the reliability of lipid peroxidation markers as indexes of oxidative damage, as higher levels of peroxidation products may simply reflect increased absolute lipid levels rather than increased free radical production or relatively higher proportion of peroxidized lipids. Such a link between circulating lipids and oxidative stress has been explored in human nutrition and medical studies (Sies et al. 2005; Halliwell and Lee 2010; Wallace et al. 2010). Also, a positive correlation between plasma MDA and triglyceride levels has been reported in a bird species (Romero-Haro and Alonso-Alvarez 2014). But surprisingly, the implications of such potential association for the reliability of oxidative stress markers have been largely overlooked.

In this study, we analyzed the relationship between two widely used markers of lipid peroxidation, MDA and ROMs, and lipid concentration in blood. We used samples from four bird species and individuals of different age (nestlings and adults). Studied species belonged to phylogenetically distant groups and were characterized by different diets, to increase the generalizability of the results. These were the granivorous red-legged partridge (*Alectoris rufa*) and zebra finch (*Taeniopygia guttata*), the omnivorous spotless starling (*Sturnus unicolor*), and the carnivorous marsh harrier (*Circus aeroginosus*) and Montagu's harrier (*Circus pygargus*). As indicators of lipid levels in blood, we used plasma triglyceride and cholesterol concentrations. Triglycerides (or triacylglycerols) are esters of glycerol with three fatty acid groups and constitute the main chemical form by which vertebrates store and transport lipids in the body (Bruss 1997; Karantonis et al. 2009). Cholesterol is a steroid molecule that plays an essential role in lipid transport, being part of lipoproteins that distribute lipids throughout the body (Bruss 1997). We predicted that levels of oxidative damage (MDA and ROMs) would be positively related to plasma levels of triglycerides and/or cholesterol, two molecules linked to lipid circulation in the bloodstream. Triglycerides are the chemical form of storage and transport of the main targets of lipid peroxidation (i.e., polyunsaturated fatty acids), so we further predicted a tighter association between lipid peroxidation marker levels and triglyceride levels as compared with cholesterol levels.

Methods

Sample Origin and Collection Procedure

Red-legged partridge and zebra finch samples came from captive populations maintained at Dehesa de Galiana experimental facilities (Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain). In May 2007, we blood sampled 36 adult nonbreeding male partridges, which were individually housed in outdoor cages with commercial pelleted food (20% protein, 4.5% fat, 3.7% cellulose; Super Feed, Madrid, Spain) and water provided ad lib. (Pérez-Rodríguez et al. 2006, 2007). In November 2012, we blood sampled 54 adult nonbreeding male zebra finches, which were housed indoor in cages with straw, a commercial mix of seeds for exotic birds (KIKI, Callosa de Segura, Spain), and water provided ad lib. (Romero-Haro and Alonso-Alvarez 2014). Wild spotless starling samples were obtained from a study colony breeding in nest boxes located in Soto del Real (Madrid; Muriel et al. 2013). Ninety-eight adult starlings were captured and sampled between March and April 2011 during the mating season (at the nest construction phase, i.e., before egg laying; Lopez-Rull and Gil 2009). The reproduction of the entire colony is monitored every season, thus allowing us to determine the exact hatching date of every nestling in the population (Muriel et al. 2013). From this study colony, in June 2010 we also blood sampled 77 14-d-old nestlings, each from a different nest. Marsh harriers and Montagu's harriers were sampled in western France, at study sites located in Charente-

Maritime (marsh harrier) and Deux-Sèvres (Montagu's harrier) districts in March–July 2010 (Sternalski et al. 2010, 2012). In each area, harrier nests were monitored to assess stage of reproduction and to determine hatching dates. Nestlings (76 marsh harriers and 87 Montagu's harriers) were sampled before fledging, at 32 and 28 d of age on average, respectively.

All blood samples were collected using heparinized syringes and were taken from the jugular vein (partridges, zebra finches, and starlings) or the brachial vein (harriers). Gauge sizes were 0.3 mm × 8 mm for zebra finches and 0.5 mm × 16 mm for partridge starlings and harriers. Samples were kept cold until reaching the lab, always within 3–6 h after collection, and they were centrifuged at 2,000–5,000 *g* (depending on the species). Plasma was separated from the cellular fraction and stored at −80°C until analysis. Samples showing any degree of hemolysis were excluded from the study.

Oxidative Stress Markers

Quantification of MDA levels in plasma was used as biomarker of lipid peroxidation in partridge, zebra finch, and starling samples, whereas ROMs were assayed on marsh harrier and Montagu's harrier samples. For MDA assays, we followed the protocol of Agarwal and Chase (2002), with modifications by Hall et al. (2010), as described previously in Romero-Haro and Alonso-Alvarez (2014). Briefly, butylated hydroxytoluene solution (0.05% w/v in 95% ethanol), 0.44 M phosphoric acid solution, and 42 mM thiobarbituric acid (TBA) solution were added to plasma samples or standards (1,1,3,3-tetraethoxypropane 5 mM serially diluted using 40% ethanol). Assay tubes were capped, vortexed, and heated at 100°C for 1 h in a dry bath incubator to allow formation of MDA-TBA adducts. The reaction was stopped by placing tubes on ice for 5 min. Subsequently, n-butanol was added to each tube to extract the MDA-TBA complex, being vortexed for 60 s and centrifuged at 18,000 *g* at 4°C for 3 min. Fifty microliters of the upper (n-butanol) phase were moved to high-performance liquid chromatography (HPLC) vials, which were immediately saturated with N_2 to avoid oxidation. Samples and standards were injected into an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) fitted with a fluorescence detector set and a 5-mm ODS-2 C-18 4.0 × 250-mm column maintained at 37°C. The mobile phase was MeOH:KH_2PO_4 (50 mM; 40:60 v/v), running isocratically for 10 min at a flow rate of 1 mL/min. Data were collected at 515 nm (excitation) and 553 nm (emission). Absolute volumes of reactives used in the assays varied among species but were always proportional to those reported in Romero-Haro and Alonso-Alvarez (2014). Repeatabilities, calculated for every species by twice assaying a subsample of 20 individuals of each, were always high ($r > 0.86$, $P < 0.001$).

ROMs were analyzed using commercial kits (d-ROMs, Diacron, Grosseto, Italy). We diluted 15 μL of plasma in a 200-μL solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen. This mixture was then incubated for 75 min at 37°C. When the metabolites react with an alkyl-substituted aromatic amine solubilized in the chromogen, they produce a complex whose color intensity is directly proportional to their concentration. After incubation, the absorbance was read with a microplate reader (Biotek Powerwave XS2, Winooski, VT) at 490 nm. Concentration of ROMs (in H_2O_2 equivalents) was calculated by comparing the absorbance of samples with a calibration standard supplied with the kit. Repeatability, estimated from a random subset of 10 samples measured twice, was high ($r = 0.92$, $P < 0.001$).

Lipid Levels in Plasma

Triglyceride and cholesterol concentrations in plasma samples were assessed following the glycerol phosphate oxidase/peroxidase method and the cholesterol esterase/oxidase/peroxidase method, respectively. Commercial kits (Biosystems, Barcelona, Spain) and manufacturer instructions were followed after adjusting reagent proportions to our sample volumes. To 5 μL of plasma or standard we added 250 μL of the specific reagent supplied with the kit and incubated for 5 min at 37°C, subsequently measuring absorbance at 500 nm. The same microplate reader mentioned above was used. Repeatabilities, calculated for every species by assaying twice a subsample of 20 individuals of each, were always high for both parameters ($r > 80$, $P < 0.001$).

Statistical Analyses

Statistical analyses were conducted using Statistica v7.0 (StatSoft, Tulsa, OK). We used Pearson's correlations to analyze the relationship between triglycerides and cholesterol and lipid peroxidation biomarkers. In order to meet normality assumptions, $\log^{10}$ transformations were applied, specifically for MDA levels in red-legged partridges, for triglyceride levels in all the studied cases except spotless starling nestlings, and for cholesterol levels in zebra finches and marsh harriers. In order to allow direct comparisons across species, we used raw data for figures. All tests were two tailed.

Results

Associations between levels of lipid peroxidation (MDA and ROMs) and lipid metabolism markers (triglycerides and cholesterol) for all study cases are shown in table 1. Plasma triglycerides were positively and significantly related to lipid peroxidation markers in all species and age groups (fig. 1), explaining between 8% and 34% of their variability. By contrast, the relationship between plasma cholesterol and lipid peroxidation marker levels was less consistent across study cases: the association was positive and significant for spotless starling nestlings only, marginally significant for zebra finches, and nonsignificant for other study cases (table 1). Circulating levels of triglycerides were positively related to cholesterol in zebra finches ($r = 0.27$, $P = 0.05$), spotless starling nestlings ($r = 0.44$, $P < 0.001$), marsh harriers ($r = 0.41$, $P < 0.001$),

Table 1: Relationships between lipid peroxidation markers (malondialdehyde [MDA] or reactive oxygen metabolites [ROMs]) and plasma levels of triglycerides and cholesterol in the studied species and age groups

			Triglycerides		Cholesterol	
Species (age)	Marker	*N*	*r*	*P*	*r*	*P*
Red-legged partridge (adults)	MDA	36	.58	<.001	−.24	.155
Zebra finch (adults)	MDA	54	.28	.039	.26	.054
Spotless starling (nestlings)	MDA	77	.33	<.01	.34	<.01
Spotless starling (adults)	MDA	98	.43	<.001	.14	.157
Marsh harrier (nestlings)	ROMs	76	.28	.015	.14	.232
Montagu's harrier (nestlings)	ROMs	87	.51	<.001	.06	.572

and Montagu's harriers ($r = 0.22$, $P = 0.04$) but not in red-legged partridges ($r = -0.20$, $P = 0.24$) and spotless starling adults ($r = -0.05$, $P = 0.62$).

Discussion

Our results support our initial hypothesis that lipid peroxidation markers (MDA and ROMs) positively covary with the concentration of lipids in blood samples. Plasma triglycerides explained between 8% and 34% of variability in lipid peroxidation markers depending on the case studied. Relationships observed were consistently positive across species of different taxa (Falconiforms, Paseriforms, Galliforms), food regimes (carnivorous, omnivorous, granivorous), and age classes (nestlings, adults) and in wild birds or those held in captive conditions. This positive covariation may therefore be a general pattern, at least among birds.

Lipid peroxidation markers were consistently associated with circulating triglyceride levels but not with cholesterol levels: a positive association was significant in spotless starling nestlings and marginally significant in zebra finches but was not found in other cases (but see also Casagrande et al. 2011 for a correlation between cholesterol and ROMs plasma levels in female but not male Eurasian kestrels *Falco tinnunculus*). There was a positive association between triglycerides and cholesterol in most study cases (four out of six), which was expected because both molecules circulate through the organism as a part of different types of lipoproteins (Bruss 1997; Karantonis et al. 2009). However, triglycerides are the main form of storage and transport of polyunsaturated fatty acids. Polyunsaturated fatty acids are the main target of lipid peroxidation in the organism, thus explaining the tighter association between lipid peroxidation markers and triglycerides than with cholesterol.

A proximate explanation for the positive relationship between triglycerides and lipid peroxidation markers would be that higher levels of circulating triglycerides (due to increased fat intake or lipid mobilization from fat stores) imply greater amounts of these oxidizable substrates in the blood (Sies et al. 2005). This would set a favorable scenario for the amplification of the chain reaction of lipid peroxidation (Halliwell and Gutteridge 2007; Mateos and Bravo 2007), increasing the production of peroxidation products originated by a given production rate of reactive oxygen species. An alternative, not mutually exclusive, explanation could be that the covariation between triglyceride and lipid peroxidation marker levels arises through the intestinal absorption of peroxidation products present in food, also positively related to absorption of dietary lipids (Cohn 2002; Sies et al. 2005). In other words, the positive correlation between lipids and peroxidation levels would already be present in the diet. This would imply that plasma relationships would not be directly the result of the interaction between free radicals and lipids in blood. However, we found the same positive correlation in partridges and zebra finches, which were feeding on commercial standardized diet, and therefore all individuals were ingesting foods with the same metabolite-to-lipid ratio. Finally, we cannot rule out the possibility that some MDA could have been formed from lipids during the heating step of the analyses (Lepenna et al. 2001), contributing to the positive relationship between MDA and lipid content of the samples. Alternative protocols for MDA quantification not involving sample heating would be helpful to discard this possibility (e.g., Karatas et al. 2002). In any case, it should be noted that the same positive correlation with lipids was found for ROMs and has also been reported for isoprostanes (Halliwell and Lee 2010), neither of which requires sample heating during analysis. Therefore, our findings do not seem to be the result of analytical artifacts.

In medicine, the focus on the association between circulating lipids and oxidative damage markers in blood is mostly on health consequences and particularly on vascular diseases (Rumley et al. 2004). Medical literature has used terms such as "postprandial" or "nutritional oxidative stress" to define this association (Sies et al. 2005; Wallace et al. 2010), thus linking this pattern to human dietary behaviors. However, the relative contribution of the two proposed mechanisms mentioned above remains unknown. Studies using lipid peroxidation markers that do not seem to be absorbed through the gastrointestinal tract in sufficient quantities to affect plasma levels, such as isoprostanes (Halliwell and Gutteridge 2007), would help disentangling both hypotheses. Interestingly, correlations between circulating levels of lipids and isoprostanes in humans have also been reported (Halliwell and Lee 2010), which may support the hypothesis that higher lipid levels promote lipid peroxidation.

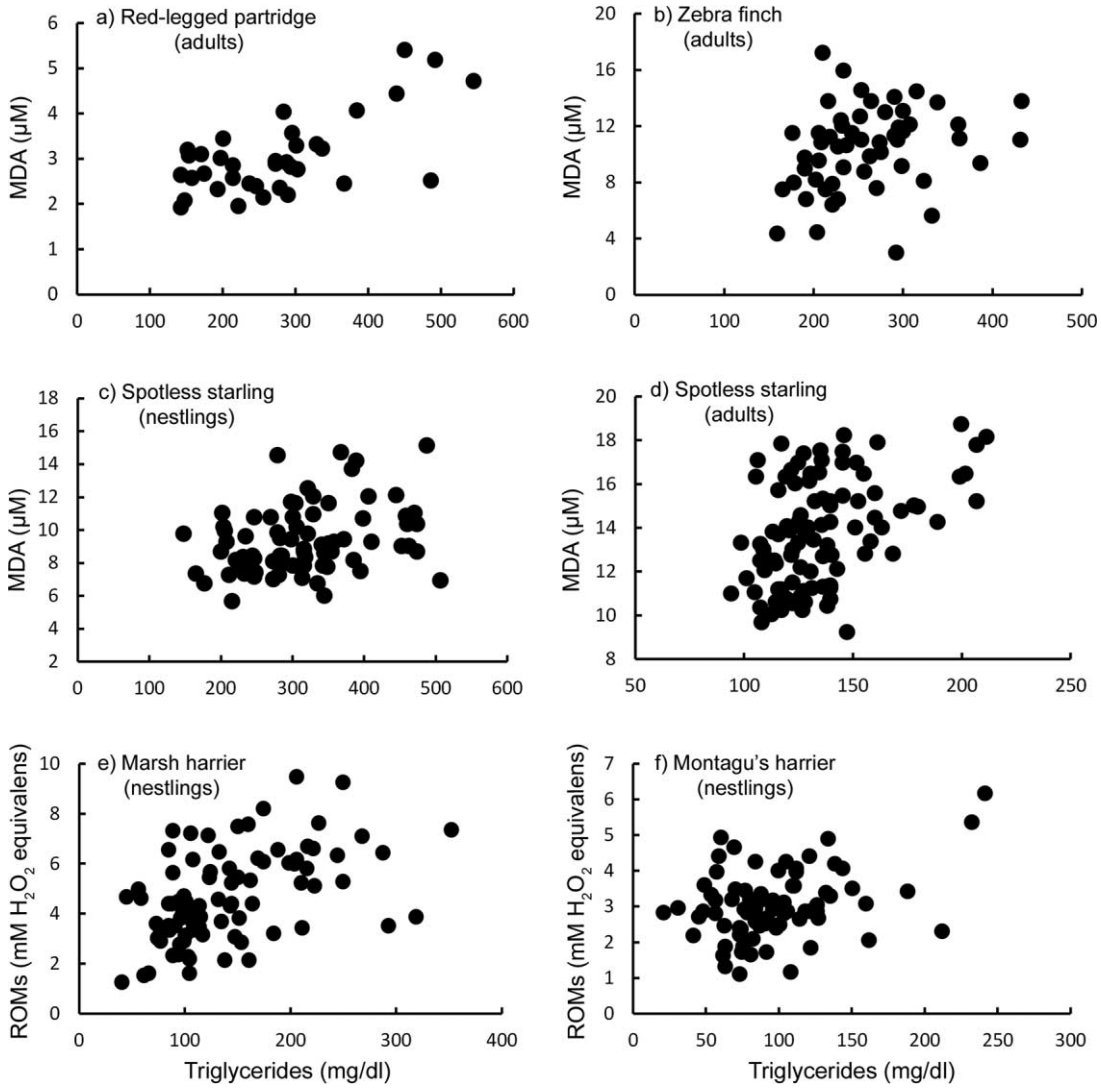

Figure 1. Relationship between oxidative stress marker levels (malondialdehyde [MDA] or reactive oxygen metabolites [ROMs]) and triglyceride levels in the six study cases.

Independently of the proximate mechanism responsible for the covariation reported here, our results could have important implications for the interpretation of lipid peroxidation marker levels, surprisingly overlooked so far (but see Halliwell and Lee 2010). Our study suggests that variations in blood lipid levels introduce undesirable bias or noise in measurements of lipid oxidative damage markers. This would lead to the equivocal inference that individuals or samples with higher lipid levels are experiencing higher levels of damage, when they can actually exhibit the same or lower overall production of free radicals. This would be particularly important in studies aimed at relating oxidative damage to traits linked to the body condition or metabolism of the individual. Fasting reduced body condition or exercise often leads to increased levels of plasma triglycerides due to lipolysis of fat stores (Jenni and Jenni-Eiermann 1992; Alonso-Alvarez and Ferrer 2001; Karantonis et al. 2009), which may in turn result in elevated lipid peroxidation markers and a wrong assessment of increased oxidative stress. Conversely, improved condition or recent food intake would result in the same effect due to lipid absorption and circulation (Jenni and Jenni-Eiermann 1992; Karantonis et al. 2009), thus leading to a—probably unexpected—positive relationship between nutritional state and oxidative damage or to an underestimation of the negative association between condition and lipid peroxidation marker levels. It cannot be discarded, however, that the effects circulating lipids on oxidative damage can be modulated by their interaction with other components of the diet (e.g., Alan and McWilliams 2013). Finally, high lipid levels may also affect the oxidative damage experienced by other biomolecules, such as proteins or DNA, by altering the oxidation cascade (see below).

The easiest way to control for variability in lipid levels would be to combine lipid peroxidation marker assays with

analyses of triglycerides. This will be feasible in most studies as available commercial kits are inexpensive and require small (ca. 5 μL) sample volumes. This will allow us to study not only variation in lipid peroxidation products per volume of sample but also variation in the proportion of peroxidation in the plasma lipid fraction. Adding lipid concentration as a covariate in the analyses seems the most correct procedure, as the use of ratios may report misleading results (Packard and Boardman 1999; Garcia-Berthou 2001). In any case, we encourage reporting the analysis of raw (i.e., uncorrected for lipid concentration) lipid peroxidation biomarker data as well (e.g., Romero-Haro and Alonso-Alvarez 2014). The simultaneous use of values of lipid peroxidation markers corrected and uncorrected for variability in circulating lipid levels would be more informative and can shed new light on our understanding of physiological processes. In other words, the problem would not be the presence of bias but the way in which we interpret our results.

ROMs are by-products of lipid peroxidation, but they also exhibit a strong prooxidant activity in the organism, and their absolute concentration can therefore be biologically meaningful to obtain a more complete picture of the ongoing redox processes in the organism. The same applies to MDA, which can exert cytotoxic and prooxidant effects, although its reactivity seems much lower than that of ROMs (Del Rio et al. 2005; Halliwell and Gutteridge 2007). Hence, uncorrected oxidative damage values could, at least in part, serve as an index of potential susceptibility to future damage, whereas lipid-corrected values may better estimate the level of damage that has already taken place (Romero-Haro and Alonso-Alvarez 2014).

Concluding, our results indicate that lipid peroxidation marker levels (MDA and ROMs) commonly used for measuring oxidative damage can be altered by the lipid content of the sample, which may affect our conclusions. Including measures of triglyceride levels and reporting both corrected and uncorrected measures of lipid peroxidation (by including or not including triglyceride levels as a covariate) would improve our understanding of the oxidative status of the individual. Future studies should clarify whether this covariation is due to the increased susceptibility of individuals circulating high lipid levels or to a higher absorption of both lipids and peroxidation products present in the diet. Finally, exploring this covariation in tissues other than blood, such as liver or muscles, and in other taxa would be helpful to determine the generality of this relationship and its implications for the study of oxidative stress in evolutionary biology and ecology.

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