1   **Oxidative stress ecology and the d-ROMs test:**

2   **facts, misfacts and an appraisal of a decade's work**

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14   **Running head:** Oxidative stress and the d-ROMs test

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Abstract In recent years, behavioural ecologists have taken to studying oxidative stress in free-ranging organisms because it has been proposed as an important mediator of life-history trade-offs. A plethora of methodological approaches to quantify biomarkers associated with oxidative stress exist, each one with its own strengths and weaknesses. The d-ROMs test has emerged as one of the favoured assays in ecological studies because of its reliability, sensitivity to specific perturbations of the organism's oxidative balance, and medical and ecological relevance. Criticisms have been, however, raised about its specificity for oxidative damage. In this article, I have reviewed basic information about the d-ROMs test, its validation, the methodological mistakes made in the studies that attempted to criticise this assay and the application of this assay to ecological studies of oxidative stress. All the direct and indirect evidence shows that the d-ROMs test is a valuable assay for the quantification of plasma or serum primary (or early) oxidative damage molecules and, possibly, of other biological matrices and provides ecologically relevant information.

Keywords Biomarker • Life-history • Oxidative damage • Physiology

Introduction

Oxidative stress is a complex multifaceted biochemical condition of cells, which occurs when there is an increased rate of oxidative molecular damage and oxidation of non-protein and protein thiols that regulate the cell oxidative balance (Sies 1991; Jones 2006; Halliwell and Gutteridge 2007). Although biomedical and toxicological sciences have traditionally centralised research on oxidative stress, in recent years, ecologists have also taken to studying oxidative stress in free-ranging organisms and have integrated principles of oxidative stress into several core evolutionary concepts, such as life-history trade-offs (e.g. survival vs. reproduction), senescence and sexual selection. It is increasingly recognised that the need to manage the oxidative status in an optimal way may be an important mechanism driving the outcome of many life-history trade-offs (Costantini 2008, 2014; Metcalfe and Alonso-Alvarez 2010; Blount et al. 2015; Tobler et al. 2015).

There are many methodological approaches for the assessment of different biomarkers of oxidative status, including assays of oxidative damage, enzymatic or non-enzymatic antioxidants and repair molecules (Dotan et al. 2004; Halliwell and Gutteridge 2007). However, there is no single biochemical metric that fulfils the need to properly quantify the organism oxidative balance (Dotan et al. 2004). It has therefore been repeatedly recommended to couple experimental manipulations with comprehensive metrics of oxidative status. In ecological research, there are also specific restrictions inherent to many research programmes, such as the availability of only small amounts of blood and the requirement of non-terminal sampling. Hence, ecologists have been mostly relying on those biomarkers of oxidative status that can be measured in blood (e.g., plasma, red blood cells). Much work has involved markers of oxidative damage, including end-

55   products of lipid peroxidation (malondialdehyde), damage to proteins (protein carbonyls) or

56   products of oxidative damage that are generated early in the oxidative cascade (organic

57   hydroperoxides). The d-ROMs test has emerged as one of the favoured assays for the

58   quantification of some aspects of the plasma oxidative status in ecological studies. The d-

59   ROMs has enabled to characterise many significant associations between plasma oxidative

60   status and either physiological or life-history traits (Table 1). However, some mistakes have

61   been made in the interpretation of what the d-ROMs actually measures. Moreover, a few

62   criticisms have been raised about the specificity of the d-ROMs. Hence, in this article, I

63   have reviewed basic information about organic hydroperoxides because these are the main

64   molecules measured by the d-ROMs; the technical aspects of the d-ROMs; the validation of

65   this assay; the methodological mistakes made in the studies that attempted to criticise this

66   assay; and the application of this assay to ecological studies of oxidative stress.

67

68   **What are organic hydroperoxides?**

69   Organic hydroperoxides derive from the oxidation of biomolecular substrates, such as

70   polyunsaturated fatty acids, cholesterol, proteins and nucleic acids, and are precursors of

71   end-products of lipid peroxidation, such as malondialdehyde, hydroxynonenal and

72   isoprostanes (Halliwell and Gutteridge 2007; Lajtha et al. 2009). Organic hydroperoxides

73   are therefore biomolecules that were damaged by free radicals and, as such, lost their

74   functionality. In plasma of vertebrates, baseline organic hydroperoxides concentrations are

75   usually below 10 µM (e.g., examples on birds and mammals in Miyazawa 1989; Gerardi et

76   al. 2002; Montgomery et al. 2011, 2012).

An important source of organic hydroperoxides is peroxidation of lipids. Reactive species are capable of abstracting a hydrogen atom from polyunsaturated fatty acids (Fig. 1), thus initiating a chain reaction known as lipid peroxidation. During this process, membrane lipids are oxidized yielding lipid organic hydroperoxides as primary products. Cells are equipped with enzymes belonging to the glutathione peroxidase family capable of reducing organic hydroperoxides to less toxic molecules. However, organic hydroperoxides can also accumulate to some extent and participate in reactions that fuel oxidative stress and increase toxicity. For example, the toxicity of organic hydroperoxides is promoted by the presence of metals which catalyse their cleavage (Fenton reaction), leading to the generation of two highly reactive and histolesive pro-oxidants, namely the alkoxyl (R-O•) and alkylperoxyl (R-OO•) radicals (Girotti 1998). Organic hydroperoxides are known to alter cell membrane fluidity and properties and to promote cell necrosis and death (Kagan et al. 2004). A wide range of organic hydroperoxides can also be formed from the reaction of proteins (Fig. 2) or nucleic acids (Fig. 3) with reactive species. For example, amino acids like proline, glutamate or lysine have been found to generate hydroperoxides (Simpson et al. 1992). Also, it has been found that reaction between hydroxyl radical and nucleic acids can generate several DNA hydroperoxides, such as the hydroxy-6-hydroperoxy-5,6-dihidrothymidine or the 6-hydroxy-5-hydroperoxide-5,6-dihydro-2'-deoxycytidine (Cadet and Di Mascio 2006; Miyamoto et al. 2007).

Assays for measuring organic hydroperoxides

There are several methodological approaches for the quantification of organic hydroperoxides in serum and plasma or in other biological matrices. These methods include
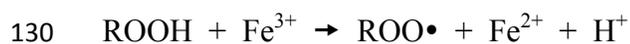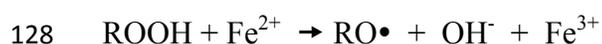
100   (i) colorimetric assays (e.g., FOX2 assay, d-ROMs assay, LPO assay kit) and flow

101   cytometry (Sirak et al. 1991) for the quantification of total or lipid hydroperoxides and (ii)

102   chromatographic techniques (e.g., gas chromatography-mass spectrometry, Kulmacz et al.

103   1990; high performance liquid chromatography with chemiluminescence detection, Ferretti

104   et al. 2005) for the specific quantification of certain groups of organic hydroperoxides. Of

105   these methods, the d-ROMs assay has been increasingly used because of its high

106   performance in terms of intra- and inter-coefficient of variation (below 10%), recovery rate

107   (e.g., between 92 and 106% in Vassalle et al. 2006 and Pasquini et al. 2008) and linearity of

108   dilution series of plasma samples (e.g., $R^2 \geq 0.95$ in Pasquini et al. 2008).
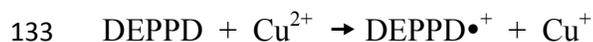
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110   **Description of the d-ROMs test**

111   The d-ROMs test measures the oxidant ability of a serum or plasma sample towards a

112   particular substance (modified aromatic amine) used as an indicator (chromogen). Organic

113   hydroperoxides are the compounds that mainly contribute to such oxidant ability, hence

114   providing an indirect estimate of organic hydroperoxides (Alberti et al. 2000). The

115   quantification of organic hydroperoxides is indirect because the acidic pH of the buffer

116   used for the reaction favours the release of metal ions like iron (Fe^{2+} and Fe^{3+}) and copper

117   (Cu^+ and Cu^{2+}) from circulating proteins. These metals catalyse the cleavage of organic

118   hydroperoxides, leading to the generation of two free radicals, i.e., RO• and ROO•, that

119   oxidize the alkylated amine DEPPD (*N,N*-diethyl-*para*-phenylendiamine) contained in the

120   chromogen solution to its red radical cation, the intensity of the resulting colour being

121   related to the amount of organic hydroperoxides present in the sample. The overall amount

122   of metal ions that can trigger the Fenton reaction occurs in excess in plasma or serum (i.e.,

123 > 10 μM; e.g., Lumeij and de Bruijne 1985; Suominen et al. 1988; Spolders et al. 2010;

124 Kautz et al. 2014) as compared to the amount of organic hydroperoxides, hence it is

125 unlikely that the availability of metal ions might limit the performance of the assay.

126 Moreover, according to the following reactions, both iron and copper ions are reduced back

127 continuously after they were oxidised, avoiding any reduction in their availability:

128 $\quad ROOH + Fe^{2+} \rightarrow RO\bullet + OH^- + Fe^{3+}$

129 $\quad ROOH + Fe^{3+} \rightarrow RO\bullet + [Fe(IV){=}O] + H^+$

130 $\quad ROOH + Fe^{3+} \rightarrow ROO\bullet + Fe^{2+} + H^+$

131 and

132 $\quad ROOH + Cu^+ \rightarrow RO\bullet + OH^- + Cu^{2+}$

133 $\quad DEPPD + Cu^{2+} \rightarrow DEPPD\bullet^+ + Cu^+$

134 The results of the d-ROMs test are expressed in arbitrary units called "Carratelli units"

135 (CARR U), where 1 CARR U is equivalent to 0.08 mg of H_2O_2/100 mL or to 0.024mM

136 H_2O_2 equivalents. Note that, for practical reasons, the value is expressed as a chemical

137 equivalence and not as a concentration. This is also because as stated in the early study

138 where the d-ROMs assay was described (Alberti et al. 2000), as well as in next studies

139 (Liang et al. 2012), while organic hydroperoxides are the main molecules detected by the d-

140 ROMs assay, other primary oxidative damage molecules may also be detected (e.g., organic

141 chloramines that derive from oxidation of protein amine groups; endoperoxides in Liang et

142 al. 2012). According to the Marcus theory (1956), chemical species with a reduction

143   potential higher than 0.3 V toward a standard hydrogen electrode should theoretically be

144   able to oxidise the DEPPD, but their contribution might be relevant as long as they occur at

145   a concentration comparable to that of organic hydroperoxides.

146   Electron paramagnetic resonance (EPR) spectroscopy is the gold standard technique

147   to detect the formation of free radicals in a biological matrix and to identify their nature. In

148   an early EPR investigation of the d-ROMs test (Alberti et al. 2000), it was possible to

149   unambiguously identify the radical cation $DEPPD^{\bullet+}$ as the species responsible for the

150   colour formation and to show that the free radicals originating from the cleavage of organic

151   hydroperoxides did lead to the formation of $DEPPD^{\bullet+}$. This early assessment of the d-

152   ROMs test was replicated by another EPR investigation, which supported the conclusion

153   that oxidants like organic hydroperoxides are the main molecules that are quantified by the

154   d-ROMs test (Liang et al. 2012). Liang et al. (2012) also suggested that other oxidative

155   damage molecules like endoperoxides contribute to the d-ROMs values.

156   It has been argued that ceruloplasmin (glycoprotein produced in the liver that carries

157   copper in blood plasma) may have some interference in the d-ROMs readings due to its

158   ferroxidase activity and to the ability of its Cu^{2+} ions to oxidize the amine DEPPD (e.g.,

159   Erel 2005; Harma et al. 2006). Another possibility is that ceruloplasmin can indirectly

160   contribute to the detection of organic hydroperoxides because this protein exerts a

161   ferroxidase activity by converting ferrous to ferric ions and the resulting Cu^+ ions are in

162   turn amenable to react with hydroperoxides to give alkoxyl radicals in a Fenton-like

163   reaction (Colombini et al. 2016). However, experiments with a ceruloplasmin inhibitor

164   (NaN_3) added in excess as compared to the ceruloplasmin concentration suggested that

165   ceruloplasmin only plays a minor role (around 7%) in determining the d-ROMs test

166   readings in human sera (Alberti et al. 2000) and even lower in other species (Table 2).

167   Although the amount of sodium azide used by Alberti et al. (2000) was chosen without

168   consideration of the affinity of sodium azide for ceruloplasmin, inhibition of ceruloplasmin

169   with another molecule (the copper chelator bathocuproine disulfonate) confirmed the

170   minimal interference of ceruloplasmin (Liang et al. 2012). If the d-ROMs assay detects

171   only ceruloplasmin as suggested by criticisms, it should be expected to find a very high R^2,

172   probably higher than 0.90, between d-ROMs values and ceruloplasmin. New recent studies

173   found a weak correlation between the results of the d-ROMs assay and the amount of

174   ceruloplasmin actually present in the samples (R^2 = 0.0009, Costantini et al. 2014a; R^2 =

175   0.18 or R^2 = 0.07 when one outlier outside the 99% confidence interval is excluded,

176   Colombini et al. 2016). Moreover, Colombini et al. (2016) showed that the addition of

177   ceruloplasmin (2.27 or 6.8 μM) to human serum samples caused on average an increase of

178   around 6% of the d-ROMs values, which is in agreement with previous work.

179   In Buonocore et al. (2000) several determinations, adding purified glutathione

180   peroxidase with reduced glutathione in excess to the plasma sample, were performed to

181   prove that the reaction of the d-ROMs test was due to peroxyl and alkoxyl radical products.

182   This procedure was used because glutathione is used by the enzyme glutathione peroxidase

183   to reduce organic hydroperoxides to their corresponding alcohols. In all determinations

184   made, a decrease of more than 90% in the signal was observed, supporting early work by

185   Alberti et al. (2000). Although glutathione is per se a scavenger of free radicals (i.e., it

186   provides its hydrogen atom to radical anions), the amine used by the d-ROMs assay is a

187   radical cation, hence it cannot accept the hydrogen atom from glutathione. Hence, a direct

188   reaction between glutathione and the amine can be excluded. This is further demonstrated

by a recent study, where experimental decrease of glutathione by administration of sulfoximine was associated with increased d-ROMs values (Costantini et al. 2016; Fig. 4). Although glutathione and d-ROMs were measured in red blood cells and plasma, respectively, note that it is well established that sulfoximine reduces glutathione synthesis in the whole body, including kidney (Griffith and Meister 1979), jejunum, lung, heart, liver and brain (Favilli et al. 1997) and plasma (Ovrebø et al. 1997; Ovrebø and Svardal 2000). Finally, note that Kilk et al. (2014) found that glutathione did not affect the d-ROMs values at normal serum values.

Indirect evidence: sensitivity of the d-ROMs assay to pro-oxidant agents

A way to test the sensitivity of an assay to perturbations of the organism's oxidative balance is to manipulate the free radical production of an organism using methods that specifically increase basal production of free radicals, such as exposure to hyperoxia or injection of generators of reactive species or of inhibitors of antioxidants. Several experimental reports have clearly shown that the d-ROMs test is very sensitive to specific manipulations of oxidative stress level. For example, during 5 and 15 minutes of reperfusion (i.e., restoration of the flow of blood to a previously ischemic tissue or organ) of Syrian hamsters, plasma values of d-ROMs increased by 72% and 89%, respectively, as compared to baseline values and declined to baseline after 30 minutes of reperfusion (Bertuglia and Giusti 2003). However, pretreatment of Syrian hamsters with the antioxidant enzyme superoxide dismutase maintained d-ROMs values at normal levels, indirectly showing that most molecules detected by the d-ROMs test come from oxidation of biomolecules induced by free radicals. Similarly, Benedetti et al. (2004) found that the

repeated exposures of patients to hyperbaric oxygen (i.e., 100% oxygen) led to a significant

increase in plasma d-ROMs values, as well as in another biomarker of oxidative damage

(thiobarbituric acid reactive substances). In another similar experiment, Nagatomo et al.

(2012) found that there were no differences in d-ROMs values in rats exposed to 14.4%,

20.9%, and 35.5% oxygen. However, d-ROMs values increased in the rats exposed to

39.8% and 62.5% oxygen. d-ROMs values were the highest in the rats exposed to 82.2%

oxygen. Morphological changes in the red blood cells induced by oxidative attack from

reactive oxygen species were also observed in the rats exposed to 39.8%, 62.5%, and

82.2% oxygen (Nagatomo et al. 2012). In another experiment, domestic canaries were

injected with DL-buthionine-(S,R)-sulfoximine, a compound that reduces cellular levels of

glutathione by inhibiting its synthesis (Griffith and Meister 1979; Bailey 1998). As

compared to controls, canaries treated with sulfoximine had a significant decrease of

reduced glutathione and a significant increase of d-ROMs values, respectively; in contrast,

the activity of ceruloplasmin was not affected by the treatment (Costantini et al. 2016; Fig.

4).

Further considerations on the d-ROMs assay

Among species variation in ceruloplasmin and d-ROMs values also provides indirect

support for the specificity of the d-ROMs assay. For example, while humans and pigs have

similar activity of ceruloplasmin (Schosinsky et al. 1974; Feng et al. 2007), d-ROMs values

in pigs are 2 to 6 times higher than in humans (humans, 325 UC $\approx$ 7.8 mM H_2O_2

equivalents in Schöttker et al. 2015b; pigs, 558 to 1750 UC $\approx$ 13.4 to 42.0 mM H_2O_2

equivalents in Brambilla et al. 2002). Similarly, while ceruloplasmin is already active and

235 detectable in young individuals at levels similar to those of adults (Lin et al. 2004; Fleming

236 et al. 2009; Wang et al. 2014), d-ROMs values may not be detectable in plasma of young

237 Eurasian kestrel individuals or are lower than those of adults (young kestrels in Costantini

238 et al. 2006; adult kestrels in Casagrande et al. 2011; Vassalle et al. 2006 for an example on

239 humans; Sgorbini et al. 2015 for an example on horses).

240 Although, in general, the patterns of variation of d-ROMs values and ceruloplasmin

241 differ between each other implying low or no correlation between them (see e.g., Assenza

242 et al. 2009; Talukder et al. 2014, 2015), it has to be considered that it is not correct to infer

243 about a potential interference of a given molecule in an assay from a simple correlation.

244 Ceruloplasmin contributes to the regulation of oxidative balance in the organism (Goldstein

245 et al. 1979; Calabrese and Carbonaro 1986; Samokyszyn et al. 1989; Ehrenwald et al.

246 1994), hence it can happen to find or not to find correlations between ceruloplasmin and

247 markers of oxidative damage, including organic hydroperoxides (Bednarek et al. 2004;

248 Maykova et al. 2013; Saravanan and Ponmurugan 2013; Kusuma Kumari and

249 Sankaranarayana 2014).

250 Results obtained with the d-ROMs test are also consistent with studies that

251 quantified organic hydroperoxides using other methods. For example, increased production

252 of organic hydroperoxides (as measured by the d-ROMs) during an immune/inflammatory

253 response is in agreement with other studies that quantified the production of organic

254 hydroperoxides using different methods (e.g., effect of 12-O-tetradecanoylphorbol-13-

255 acetate on organic hydroperoxides in Sirak et al. 1991; effect of lipopolysaccharide on

256 organic hydroperoxides in Riedel et al. 2003; effect of multiple sclerosis on organic

257 hydroperoxides in Ferretti et al. 2005). This strong and significant link between organic

258 hydroperoxides and immune response is further corroborated by the significant correlations

259 that have been found between d-ROMs values and leucocyte counts or antibody titres (e.g.,

260 van de Crommenacker 2011; Casagrande et al. 2012; Schneeberger et al. 2013). Note also

261 that injection of pigeons with *Escherichia coli* lipopolysaccharide caused inflammation, but

262 did not induce any relevant changes in ceruloplasmin (Dudek et al. 2013). Increased

263 production of organic hydroperoxides was also found in relation to increased levels of

264 stress hormones using either the d-ROMs (Costantini et al. 2008; Haussmann et al. 2012) or

265 other assays (Sato et al. 2010; Balkaya et al. 2011).

266 Some authors used the d-ROMs assay to estimate production of free radicals (Al-

267 Johany et al. 2009; Noguera et al. 2011). Organic hydroperoxides are likely to better reflect

268 the basal free radical production than endproducts of lipid peroxidation (e.g.,

269 malondialdehyde). This is because organic hydroperoxides are generated earlier in the

270 oxidative cascade than endproducts. However, organic hydroperoxides are primary

271 oxidative damage molecules and none studies have tested the correlation between organic

272 hydroperoxides and amount of free radicals produced. The correlation between free radical

273 generation and organic hydroperoxides is unlikely to be very strong because there are

274 various mechanisms and molecules that either prevent oxidation of biomolecules or remove

275 organic hydroperoxides from the organism. It is therefore premature to infer about the

276 production of free radicals from the results of the d-ROMs assay.

277 It has also been suggested that the d-ROMs assay is a measure of potential damage

278 (Stier et al. 2015). This sentence implies that the d-ROMs test detects molecules that may

279 potentially cause damage. This may certainly happen because molecules like organic

280 hydroperoxides can be cleaved into free radicals, so fuelling the oxidative cascade.

281 However, it is unknown the extent to which this can happen (e.g., peroxidases reduce

282 organic hydroperoxides to their corresponding alcohols before they can be cleaved). It

283 therefore appears more correct to refer to oxidative damage because this is what the main

284 molecules detected by the d-ROMs assay are. Note that, although peroxidases interact with

285 organic hydroperoxides in the organism, it is unlikely that peroxidases cause significant

286 interference with the d-ROMs test because of the low activity of peroxidases in plasma and

287 the weak correlation between peroxidase activity and d-ROMs values (Costantini et al.

288 2011, 2012a).

289

290 **Beyond the blood: application of the d-ROMs to other biological matrices**

291 Although the application of the d-ROMs test has been originally assessed for blood, recent

292 studies showed that the d-ROMs test might also be applied to other biological matrices. For

293 example, Castellini et al. (2003) found that d-ROMs values of seminal plasma of rabbits are

294 correlated to those measured in blood plasma. Ito et al. (2009) found that plasma d-ROMs

295 values were positively and significantly correlated ($r = 0.50$) with urine d-ROMs values. In

296 another study, the d-ROMs test was applied to immune cells. Specifically, bovine

297 peripheral blood mononuclear cells (component of the immune system) had been isolated

298 from the whole blood and were then incubated for 2 and 7 days at different concentrations

299 of mycotoxins to stimulate their activity (Bernabucci et al. 2011). Compared with the

300 control, an increase of intracellular d-ROMs values (and also of malondialdehyde, which is

301 an endproduct of lipid peroxidation) was observed, indicating the high sensitivity of the d-

302 ROMs assay to perturbations of the oxidative balance caused by an immune response.

303   Similarly, in vitro induction of oxidative stress in prostate cancer cells resulted in

304   the increase of d-ROMs values in the extracellular compartment, and of reactive oxygen

305   species and DNA damage in the intracellular compartment (Tomasetti et al. 2010).

306   Finally, Criscuolo et al. (2010) found that the d-ROMs test may also be applied to

307   haemolymph of invertebrates. Criscuolo et al. (2010) found that the d-ROMs values were

308   higher in the haemolymph of short-lived male tarantulas (which also had higher superoxide

309   production and lower antioxidant defences) than in the haemolymph of their long-lived

310   females (which also had lower superoxide production and higher antioxidant defences).

311

312   **Does the d-ROMs assay provide ecologically relevant information?**

313   Medical and veterinary research showed that the d-ROMs test has a significant diagnostic

314   value of many pathological statuses, in agreement with other biomarkers of oxidative

315   damage, such as the gold standard "isoprostanes" (e.g., correlation between d-ROMs values

316   and isoprostanes = 0.68 in Lubrano et al. 2002). For example, d-ROMs values were found

317   to be significantly associated with mortality in humans independently from established risk

318   factors, including inflammation (Schöttker et al. 2015a).

319   It is now increasingly recognised that the d-ROMs is also a valuable test for the

320   investigation of oxidative stress in ecological studies. Work has, for example, shown that d-

321   ROMs values are associated with behavioural or fitness-related traits or reflect potential

322   physiological costs induced by short-term activation of immune response or changes in the

323   hormonal profile (Table 1). For example, experimental increase of plasma d-ROMs values

324   was found to delay egg laying and reduce clutch size in a songbird (Costantini et al. 2016),

325   which are two important fitness-related traits under female control. High plasma d-ROMs

326   values were found to be associated with reduced survival perspectives in two seabird

327   species (Costantini et al. 2015; Herborn et al. 2016). Geiger et al. (2012) and Hau et al.

328   (2015) also found that high plasma d-ROMs values were associated with shorter telomeres,

329   which is an emerging marker of disease risk and biological ageing. Finally, a number of

330   studies found that the d-ROMs test may inform about environmental quality (e.g.,

331   contamination level in Bonisoli-Alquati et al. 2010; food availability in van de

332   Crommenacker et al. 2011a), adaptation to urbanisation (Lucas and French 2012;

333   Costantini et al. 2014b) or variation in behavioural phenotype (Herborn et al. 2011;

334   Costantini et al. 2012b).

335

336   **Criticisms on the d-ROMs assay are based on methodological inadequacy**

337   A few authors raised critics to the d-ROMs assay, claiming that it is not a valid assay for

338   the quantification of plasma oxidative status, organic hydroperoxides in particular.

339   However, these critics have been based on serious methodological inadequacy and,

340   importantly, on lack of respect of the protocols that have previously been validated.

341   Early criticisms about the lack of specificity were based on several mistakes done in

342   the application of the d-ROMs assay (Erel 2005; Harma et al. 2006). For example, Erel

343   (2005) used chelants for the preparation of samples, which interfere with the d-ROMs

344   reaction (Banfi et al. 2006). Chelants sequester iron ions, which are needed for the reaction

345   of the d-ROMs test and this was already explained in the validation study (Alberti et al.

346   2000). Hence, anticoagulants like EDTA or citrate should not be used.

347   Recently, similar critics to those of Harma et al. (2006) were surprisingly raised by

348   Kilk et al. (2014). Again, these critics have been based on methodological inadequacy in

349   the application of the d-ROMs assay. First, Kilk et al. (2014) tested the capacity of the d-

350   ROMs assay to assess oxidative damage using solutions of hydrogen peroxide. They found

351   that the d-ROMs was able to weakly detect hydrogen peroxide only when it occurred at

352   very high concentrations ($\geq$ 50 μM) that are sometimes found in the plasma of individuals

353   under severe oxidative stress. Hence, the authors concluded that the amount of peroxides in

354   the plasma is a bit above the detection limit of the d-ROMs assay only under conditions of

355   strong oxidative stress. The small capacity of the d-ROMs to measure hydrogen peroxide

356   is, however, not surprising nor is it novel (see Liang et al. 2012) because the d-ROMs

357   mainly measures organic hydroperoxides (e.g., Alberti et al. 2000; Liang et al. 2012;

358   Colombini et al. 2016). The d-ROMs can detect organic hydroperoxides at concentrations

359   well below 10 μM (Fig. 5), which have been found in the plasma of several vertebrates

360   (e.g., Miyazawa 1989; Gerardi et al. 2002; Montgomery et al. 2011, 2012). For example,

361   Gerardi et al. (2002) found a significant positive correlation between d-ROMs values and

362   lipid hydroperoxides measured with the FOX2 assay.

363   Second, Kilk et al. (2014) found that d-ROMs readings of solutions containing

364   ceruloplasmin and of sera decreased by decreasing the incubation temperature from 37ºC

365   (protocol of d-ROMs) to 23ºC and to 4ºC, while those of solutions containing hydrogen

366   peroxide were less dependent on temperature. These results are again unreliable because (i)

367   the d-ROMs assay is poorly sensitive to hydrogen peroxide, (ii) the Fenton reaction is

368   highly dependent on temperature (e.g., Neyens and Baeyens 2003; Lee and Yoon 2004;

369   Hussain et al. 2014) as also shown by the decrease of the readings of pure solutions of

370   cumene hydroperoxide with the decrease of incubation temperature (Table 3), and (iii) the

371   decrease of absorbance of pure solutions of cumene hydroperoxide with temperature is

372 similar to that of plasma samples (Table 3; note that the pure solution of organic

373 hydroperoxides has to have an absorbance at 37ºC similar to that of plasma samples in

374 order to avoid "the regression to the mean effect", i.e., when the change in absorbance is

375 dependent on the initial absorbance), while that of ceruloplasmin shown in Kilk et al.

376 (2014) is not.

377   Third, the authors speculated that thiols like albumin and glutathione might interfere

378 with the assay. However, the amount of either albumin or glutathione that Kilk et al. (2014)

379 added to the serum samples was pharmacological, while, as stressed by the same authors,

380 glutathione did not affect the d-ROMs values at normal serum values.

381   Fourth, given this apparent temperature effect, the authors stated that they carried

382 out the incubation at 23ºC. Although the early assessment of the d-ROMs performance was

383 carried out at room temperature (Alberti et al. 2000), the decrease of Fenton reaction with

384 incubation temperature shown in Table 3 suggests that several of the conclusions made by

385 the authors should be taken cautiously.

386   Fifth, the supposed correlation between d-ROMs values and activity of

387 ceruloplasmin shown in figure 7 is not reliable because (i) it is an artifact of plotting two

388 species having different levels of both d-ROMs values and ceruloplasmin and (ii) more

389 importantly because the values of absorbance of sera reported in figure 7 are not compatible

390 with the normal absorbance values of the assay (see e.g. values of absorbance in Table 2 for

391 a volume of 200 μl within a plate well).

392

393 **Improvements in the application of the d-ROMs assay**

394   The d-ROMs assay can be performed using either cuvettes or well plates. The use of well

395   plates may face the experimenter with an issue when there is any formation of precipitate

396   on the bottom of the well. To overcome this problem, incubation can be done in tubes and,

397   straightaway the end of incubation, tubes can be centrifuged and the supernatant used for

398   the readings.   Another problem may be with the plasma colour when this is very yellow. In

399   this case, it is important to increase the wavelength at which readings are taken (e.g., 505

400   nm or even more), considering that, for example, one peak of absorbance of lutein is at ≈

401   476 nm. As with the plasma colour, it should also be paid attention to whether plasma

402   samples look red because of haemolysis. Iamele et al. (2002) found that a concentration of

403   haemoglobin above 0.068 mM in human serum may interfere with the d-ROMs

404   measurements.

405   The d-ROMs assay may not be sensitive, especially for young individuals, whose d-

406   ROMs values may be low (Costantini et al. 2006). In order to improve the performance of

407   the assay, it can be used more plasma than usual, but carefulness is needed in order to avoid

408   any interference of plasma colour or alterations of the pH of the buffer.

409   Finally, it has been suggested that it might be interesting to measure fatty acids

410   and/or cholesterol in plasma and to express d-ROMs values also per unit of fatty acids

411   (Pérez-Rodríguez et al. 2015). This is because fatty acids and cholesterol are substrates for

412   formation of organic hydroperoxides. Note that caution is needed because d-ROMs values

413   do not only refer to lipid hydroperoxides but also to those formed from oxidation of other

414   substrates and the correlations between d-ROMs values and either fatty acids or cholesterol

415   are often low (e.g., Casagrande et al. 2011; Kotani et al. 2013). It might, however, be

416 interesting to assess if, together with uncorrected values, d-ROMs values corrected for the

417 number of substrates that occur in plasma also provide valuable biological information.

418

419 **Conclusions**

420 Conversely to many colorimetric assays, the d-ROMs test has been properly assessed using

421 electron paramagnetic resonance. All the direct and indirect evidence shows that the d-

422 ROMs test is a valuable assay for the quantification of the plasma or serum oxidative status

423 (higher values indicating higher oxidative damage), due to primary oxidation products of

424 biomolecules (mainly organic hydroperoxides, but also endoperoxides and organic

425 chloramines). Moreover, values of d-ROMs show significant individual repeatability over

426 time (e.g., Costantini et al. 2007; Hau et al. 2015; Herborn et al. 2016). Experimental data

427 also show that this assay is very sensitive to specific pro-oxidant agents and can provide

428 ecologically valuable information, suggesting that this test might serve as a blood-derived

429 biomarker to assess the impact of oxidative damage on health and fitness perspectives in

430 animals. Clearly, as for any other metric of oxidative damage, the d-ROMs test should also

431 be used in combination with other assays in order to better assess the individual oxidative

432 balance. It is recommended that ecologists, interested in pursuing research in oxidative

433 stress ecology, get sufficient basic background in biochemistry before making their own

434 choice about what assay is suitable for their work.

435

441

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739   complex on sow reproductive performance and iron status of nursing pigs. Livest Sci

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741

742   Table 1 Examples of how changes of d-ROMs values in relation to a number of behavioural

743   traits or stressors have been found across a broad variety of organisms

Trait or stressor	Taxon	Effect	Study
Dominance status during the breeding season	*Acrocephalus sechellensis*	Dominant males had higher d-ROMs values	van de Crommenacker et al. 2011b
Dominance status during the breeding season	*Mandrillus sphinx*	High-ranking males had higher d-ROMs values	Beaulieu et al. 2014
Food habits	33 bat species	Species with a frugivorous diet had the lowest d-ROMs values, followed by omnivorous and animalivorous species	Schneeberger et al. 2014
Food habits	*Pygoscelis papua*	Colonies with the highest δ13C and δ15N values had the highest d-ROMs values	Beaulieu et al. 2015
Heat stress	*Taeniopygia guttata*	Increase of d-ROMs values	Costantini et al. 2012a
Immune response	*Falco tinnunculus*	Increase of d-ROMs values	Costantini and Dell'Omo 2006
Immune response	*Carollia perspicillata*	Increase of d-ROMs values	Schneeberger et al. 2013
Malaria infection	*Parus major*	Increase of d-ROMs values	Isaksson et al. 2013
Solicitation display	*Hirundo rustica*	Begging bout duration was negatively predicted by d-ROMs values but only after food deprivation.	Boncoraglio et al. 2012
Song behaviour	*Sturnus vulgaris*	Decrease of song rate with increase of d-ROMs values	Casagrande et al. 2014

744

745 Table 2 Comparison of absorbance values between samples that were either not treated or

746 treated with sodium azide (inhibitor of ceruloplasmin activity). Affinity of sodium azide for

747 ceruloplasmin (and so its capacity to inhibit ceruloplasmin activity) varies across species,

748 but a concentration of 1 mM of sodium azide in a buffer with a 7.4 pH has been shown to

749 reduce ceruloplasmin activity in both humans and chickens (Musci et al. 1999). The

750 capacity of sodium azide to inhibit ceruloplasmin was also suggested to increase with the

751 decrease of pH (Musci et al. 1999). This is important because the buffer used for the d-

752 ROMs assay has a pH of 4.8, hence much lower than that used in Musci et al. (1999). Data

753 on *Phalacrocorax aristotelis* are from Herborn et al. 2016. Values are expressed as mean ±

754 standard deviation. Coefficient of variation refers to variation in absorbance values between

755 samples that were either not treated or treated with sodium azide. Difference in absorbance

756 value mean between samples that were either not treated or treated with sodium azide is

757 also shown as percentage

Species	No sodium azide	1 mM sodium azide	Coefficient of variation	Difference expressed as %
Serinus canaria	0.132±0.015	0.135±0.017	3.03±1.75	+3.68
Sturnus vulgaris	0.158±0.021	0.163±0.013	4.81±1.34	+2.55
Taeniopygia guttata	0.182±0.053	0.186±0.052	2.20±1.30	+1.88
Calonectris diomedea	0.110±0.009	0.114±0.009	2.66±2.50	+3.95
Phalacrocorax	0.183±0.025	0.175±0.019	4.90±2.50	-4.37

aristotelis				

758

759

760 Table 3 Percentage of decrease of absorbance at 24 and 4°C as compared to 37°C, which is

761 the temperature required by the d-ROMs protocol. Note that the among sample variation is

762 to be expected given that in plasma samples there are several groups of organic

763 hydroperoxides that contribute to the reading and these differ in concentration among

764 individuals. Moreover, in plasma samples there are other primary oxidative damage

765 molecules that contribute to the reading (e.g., organic chloramines that derive from

766 oxidation of proteic amine groups; endoperoxides; Alberti et al. 2000; Liang et al. 2012)

767

768

	% of decrease of absorbance at 24°C as compared to 37°C	% of decrease of absorbance at 4°C as compared to 37°C
Cumene hydroperoxide 1	39	62
Cumene hydroperoxide 2	41	61
Standard 1	46	71
Standard 2	50	69
Plasma 1	42	60
Plasma 2	34	54
Plasma 3	36	57
Plasma 4	38	56
Coefficient of variation (%)	12.9	9.9

769

Figure captions

Fig. 1 Scheme illustrating the generation and fate of lipid hydroperoxides in cell membranes. PHGPx = phospholipid glutathione peroxidase; PLA2 = phospholipase A2; LOOH = fatty acid hydroperoxides; GPx = glutathione peroxidase; LOO• = fatty acid peroxyl radical; LO• = fatty acid alcoxyl radical; $^{1}O_2$ = singlet oxygen; HOCl = hypochlorous acid; $ONOO^{-}$ = peroxynitrite. Russell mechanism refers to the reaction between peroxyl radicals which generates singlet oxygen. Reprinted from Miyamoto et al. (2007) with permission from John Wiley and Sons

Fig. 2 (A) Generation of hydroperoxides in protein backbone mediated by •OH attack. (B) Amino acid hydroperoxides containing hydrogen-α. Valine hydroperoxide (Val-OOH), lysine hydroperoxide (Lys-OOH) and leucine hydroperoxide (Leu-OOH). Reprinted from Miyamoto et al. (2007) with permission from John Wiley and Sons

Fig. 3 Structures of thymidine and cytidine hydroperoxides formed by reaction of thymidine and cytidine with •OH, respectively. Reprinted from Miyamoto et al. (2007) with permission from John Wiley and Sons

Fig. 4 Pre- and post-manipulation levels of red blood cell reduced glutathione, plasma d-ROMs values and plasma ceruloplasmin of canaries in relation to injection of sulfoximine. The experimental treatment was able to decrease red blood cell concentration of reduced glutathione and to increase plasma d-ROMs values, while it did not affect activity of ceruloplasmin. Although glutathione concentration was quantified within red blood cells, it is well established that sulfoximine is a potent inhibitor of glutathione synthesis in many body compartments, plasma included (see text). Means that do not share the same letter are significantly different from each other (Tukey, $P < 0.05$). Data are shown as mean ± standard error. Data of d-ROMs and reduced glutathione are reprinted from Costantini et al. (2016) with slight modifications with permission from John Wiley and Sons

Fig. 5 Dose-response of the d-ROMs test to pure solutions of cumene hydroperoxide. The concentrations of cumene hydroperoxides used in this trial are in the range of circulating organic hydroperoxides that occur in vertebrates. The concentrations of cumene hydroperoxides refer to those of the original samples, i.e., before they are 100 fold diluted when added to the d-ROMs buffer
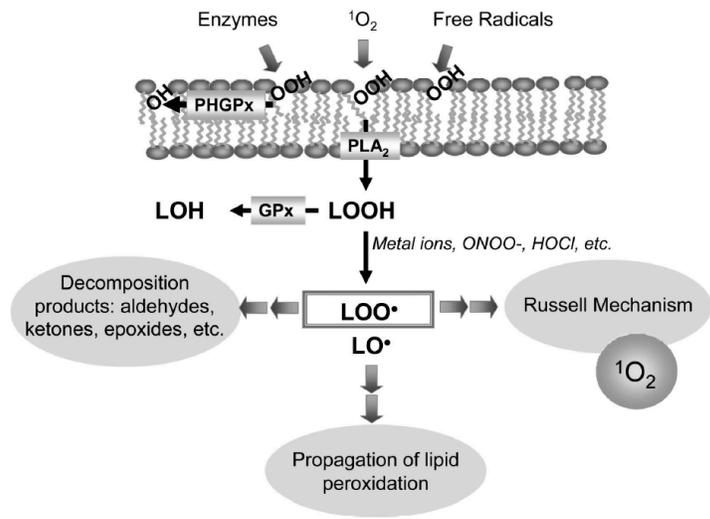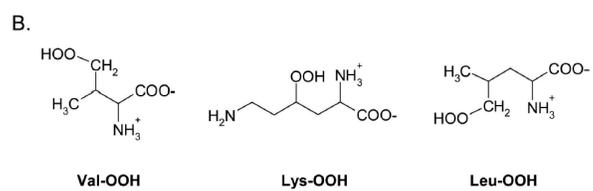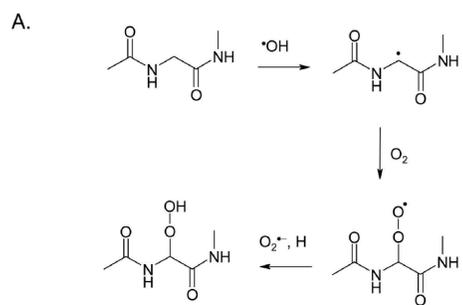
805

806

807   Figure 1

808

A.

B.

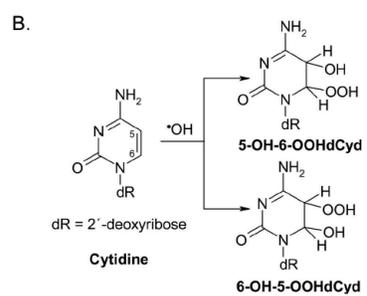**Val-OOH**   **Lys-OOH**   **Leu-OOH**
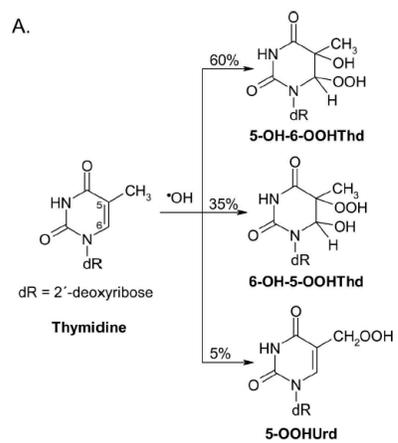
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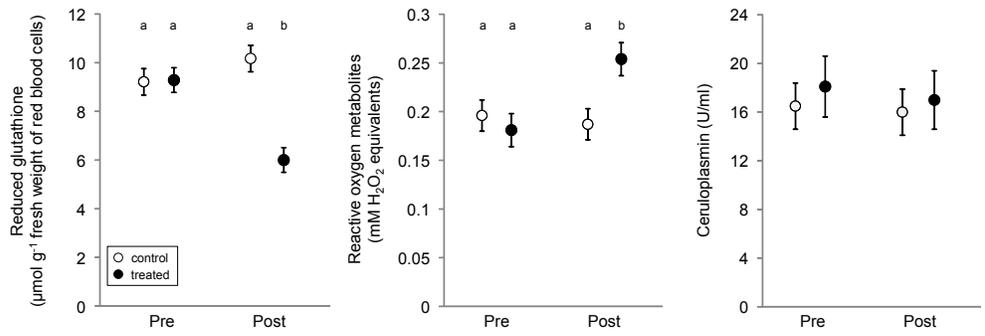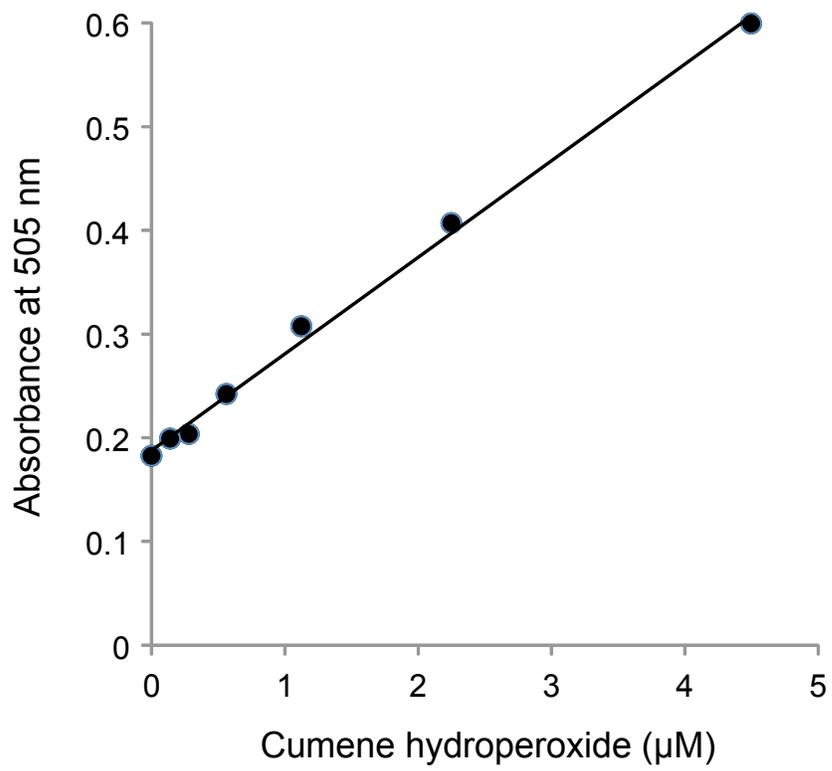811   Figure 2

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815 Figure 3

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819   Figure 4

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823   Figure 5

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