
There may be differences between this version and the published version. You are advised to consult the publisher’s version if you wish to cite from it.

http://eprints.gla.ac.uk/120076/

Deposited on: 14 July 2016
Oxidative stress ecology and the d-ROMs test:
facts, misfacts and an appraisal of a decade’s work

David Costantini

Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
Institute of Biodiversity, Animal Health and Comparative Medicine, School of Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Email: davidcostantini@libero.it

Running head: Oxidative stress and the d-ROMs test
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-
products of lipid peroxidation (malondialdehyde), damage to proteins (protein carbonyls) or products of oxidative damage that are generated early in the oxidative cascade (organic hydroperoxides). The d-ROMs test has emerged as one of the favoured assays for the quantification of some aspects of the plasma oxidative status in ecological studies. The d-ROMs has enabled to characterise many significant associations between plasma oxidative status and either physiological or life-history traits (Table 1). However, some mistakes have been made in the interpretation of what the d-ROMs actually measures. Moreover, a few criticisms have been raised about the specificity of the d-ROMs. Hence, in this article, I have reviewed basic information about organic hydroperoxides because these are the main molecules measured by the d-ROMs; the technical aspects of the d-ROMs; the validation of this assay; 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.

What are organic hydroperoxides?

Organic hydroperoxides derive from the oxidation of biomolecular substrates, such as polyunsaturated fatty acids, cholesterol, proteins and nucleic acids, and are precursors of end-products of lipid peroxidation, such as malondialdehyde, hydroxynonenal and isoprostanes (Halliwell and Gutteridge 2007; Lajtha et al. 2009). Organic hydroperoxides are therefore biomolecules that were damaged by free radicals and, as such, lost their functionality. In plasma of vertebrates, baseline organic hydroperoxides concentrations are usually below 10 μM (e.g., examples on birds and mammals in Miyazawa 1989; Gerardi et 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 alkoxy (R-O•) and alkylperoxy (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'-deoxyctydine (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
(i) colorimetric assays (e.g., FOX2 assay, d-ROMs assay, LPO assay kit) and flow cytometry (Sirak et al. 1991) for the quantification of total or lipid hydroperoxides and (ii) chromatographic techniques (e.g., gas chromatography-mass spectrometry, Kulmacz et al. 1990; high performance liquid chromatography with chemiluminescence detection, Ferretti et al. 2005) for the specific quantification of certain groups of organic hydroperoxides. Of these methods, the d-ROMs assay has been increasingly used because of its high performance in terms of intra- and inter-coefficient of variation (below 10%), recovery rate (e.g., between 92 and 106% in Vassalle et al. 2006 and Pasquini et al. 2008) and linearity of dilution series of plasma samples (e.g., $R^2 \geq 0.95$ in Pasquini et al. 2008).

**Description of the d-ROMs test**

The d-ROMs test measures the oxidant ability of a serum or plasma sample towards a particular substance (modified aromatic amine) used as an indicator (chromogen). Organic hydroperoxides are the compounds that mainly contribute to such oxidant ability, hence providing an indirect estimate of organic hydroperoxides (Alberti et al. 2000). The quantification of organic hydroperoxides is indirect because the acidic pH of the buffer used for the reaction favours the release of metal ions like iron ($\text{Fe}^{2+}$ and $\text{Fe}^{3+}$) and copper ($\text{Cu}^{+}$ and $\text{Cu}^{2+}$) from circulating proteins. These metals catalyse the cleavage of organic hydroperoxides, leading to the generation of two free radicals, i.e., $\text{RO}^\bullet$ and $\text{ROO}^\bullet$, that oxidize the alkylated amine DEPPD ($N,N$-diethyl-para-phenylenediamine) contained in the chromogen solution to its red radical cation, the intensity of the resulting colour being related to the amount of organic hydroperoxides present in the sample. The overall amount of metal ions that can trigger the Fenton reaction occurs in excess in plasma or serum (i.e.,
> 10 μM; e.g., Lumeij and de Bruijne 1985; Suominen et al. 1988; Spolders et al. 2010; Kautz et al. 2014) as compared to the amount of organic hydroperoxides, hence it is unlikely that the availability of metal ions might limit the performance of the assay. Moreover, according to the following reactions, both iron and copper ions are reduced back continuously after they were oxidised, avoiding any reduction in their availability:

\[
ROOH + \text{Fe}^{2+} \rightarrow RO^\cdot + \text{OH}^- + \text{Fe}^{3+}
\]

\[
ROOH + \text{Fe}^{3+} \rightarrow RO^\cdot + [\text{Fe(IV)}=\text{O}] + \text{H}^+
\]

\[
ROOH + \text{Fe}^{3+} \rightarrow \text{ROO}^\cdot + \text{Fe}^{2+} + \text{H}^+
\]

\[
\text{DEPPD} + \text{Cu}^{2+} \rightarrow \text{DEPPD}^\cdot + \text{Cu}^+
\]

The results of the d-ROMs test are expressed in arbitrary units called “Carratelli units” (CARR U), where 1 CARR U is equivalent to 0.08 mg of H₂O₂/100 mL or to 0.024 mM H₂O₂ equivalents. Note that, for practical reasons, the value is expressed as a chemical equivalence and not as a concentration. This is also because as stated in the early study where the d-ROMs assay was described (Alberti et al. 2000), as well as in next studies (Liang et al. 2012), while organic hydroperoxides are the main molecules detected by the d-ROMs assay, other primary oxidative damage molecules may also be detected (e.g., organic chloramines that derive from oxidation of protein amine groups; endoperoxides in Liang et al. 2012). According to the Marcus theory (1956), chemical species with a reduction
potential higher than 0.3 V toward a standard hydrogen electrode should theoretically be able to oxidise the DEPPD, but their contribution might be relevant as long as they occur at a concentration comparable to that of organic hydroperoxides.

Electron paramagnetic resonance (EPR) spectroscopy is the gold standard technique to detect the formation of free radicals in a biological matrix and to identify their nature. In an early EPR investigation of the d-ROMs test (Alberti et al. 2000), it was possible to unambiguously identify the radical cation DEPPD$^{+}$ as the species responsible for the colour formation and to show that the free radicals originating from the cleavage of organic hydroperoxides did lead to the formation of DEPPD$^{+}$. This early assessment of the d-ROMs test was replicated by another EPR investigation, which supported the conclusion that oxidants like organic hydroperoxides are the main molecules that are quantified by the d-ROMs test (Liang et al. 2012). Liang et al. (2012) also suggested that other oxidative damage molecules like endoperoxides contribute to the d-ROMs values.

It has been argued that ceruloplasmin (glycoprotein produced in the liver that carries copper in blood plasma) may have some interference in the d-ROMs readings due to its ferroxidase activity and to the ability of its Cu$^{2+}$ ions to oxidize the amine DEPPD (e.g., Erel 2005; Harma et al. 2006). Another possibility is that ceruloplasmin can indirectly contribute to the detection of organic hydroperoxides because this protein exerts a ferroxidase activity by converting ferrous to ferric ions and the resulting Cu$^{+}$ ions are in turn amenable to react with hydroperoxides to give alkoxyl radicals in a Fenton-like reaction (Colombini et al. 2016). However, experiments with a ceruloplasmin inhibitor (NaN$_3$) added in excess as compared to the ceruloplasmin concentration suggested that ceruloplasmin only plays a minor role (around 7%) in determining the d-ROMs test
readings in human sera (Alberti et al. 2000) and even lower in other species (Table 2). Although the amount of sodium azide used by Alberti et al. (2000) was chosen without consideration of the affinity of sodium azide for ceruloplasmin, inhibition of ceruloplasmin with another molecule (the copper chelator bathocuproine disulfonate) confirmed the minimal interference of ceruloplasmin (Liang et al. 2012). If the d-ROMs assay detects only ceruloplasmin as suggested by criticisms, it should be expected to find a very high $R^2$, probably higher than 0.90, between d-ROMs values and ceruloplasmin. New recent studies found a weak correlation between the results of the d-ROMs assay and the amount of ceruloplasmin actually present in the samples ($R^2 = 0.0009$, Costantini et al. 2014a; $R^2 = 0.18$ or $R^2 = 0.07$ when one outlier outside the 99% confidence interval is excluded, Colombini et al. 2016). Moreover, Colombini et al. (2016) showed that the addition of ceruloplasmin (2.27 or 6.8 µM) to human serum samples caused on average an increase of around 6% of the d-ROMs values, which is in agreement with previous work.

In Buonocore et al. (2000) several determinations, adding purified glutathione peroxidase with reduced glutathione in excess to the plasma sample, were performed to prove that the reaction of the d-ROMs test was due to peroxyl and alkoxyl radical products. This procedure was used because glutathione is used by the enzyme glutathione peroxidase to reduce organic hydroperoxides to their corresponding alcohols. In all determinations made, a decrease of more than 90% in the signal was observed, supporting early work by Alberti et al. (2000). Although glutathione is per se a scavenger of free radicals (i.e., it provides its hydrogen atom to radical anions), the amine used by the d-ROMs assay is a radical cation, hence it cannot accept the hydrogen atom from glutathione. Hence, a direct 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 ≈ 7.8 mM H₂O₂ equivalents in Schöttker et al. 2015b; pigs, 558 to 1750 UC ≈ 13.4 to 42.0 mM H₂O₂ equivalents in Brambilla et al. 2002). Similarly, while ceruloplasmin is already active and
detectable in young individuals at levels similar to those of adults (Lin et al. 2004; Fleming et al. 2009; Wang et al. 2014), d-ROMs values may not be detectable in plasma of young Eurasian kestrel individuals or are lower than those of adults (young kestrels in Costantini et al. 2006; adult kestrels in Casagrande et al. 2011; Vassalle et al. 2006 for an example on humans; Sgorbini et al. 2015 for an example on horses).

Although, in general, the patterns of variation of d-ROMs values and ceruloplasmin differ between each other implying low or no correlation between them (see e.g., Assenza et al. 2009; Talukder et al. 2014, 2015), it has to be considered that it is not correct to infer about a potential interference of a given molecule in an assay from a simple correlation. Ceruloplasmin contributes to the regulation of oxidative balance in the organism (Goldstein et al. 1979; Calabrese and Carbonaro 1986; Samokyszyn et al. 1989; Ehrenwald et al. 1994), hence it can happen to find or not to find correlations between ceruloplasmin and markers of oxidative damage, including organic hydroperoxides (Bednarek et al. 2004; Maykova et al. 2013; Saravanan and Ponmurugan 2013; Kusuma Kumari and Sankaranarayana 2014).

Results obtained with the d-ROMs test are also consistent with studies that quantified organic hydroperoxides using other methods. For example, increased production of organic hydroperoxides (as measured by the d-ROMs) during an immune/inflammatory response is in agreement with other studies that quantified the production of organic hydroperoxides using different methods (e.g., effect of 12-O-tetradecanoylphorbol-13-acetate on organic hydroperoxides in Sirak et al. 1991; effect of lipopolysaccharide on organic hydroperoxides in Riedel et al. 2003; effect of multiple sclerosis on organic hydroperoxides in Ferretti et al. 2005). This strong and significant link between organic
hydroperoxides and immune response is further corroborated by the significant correlations that have been found between d-ROMs values and leucocyte counts or antibody titres (e.g., van de Crommenacker 2011; Casagrande et al. 2012; Schneeberger et al. 2013). Note also that injection of pigeons with *Escherichia coli* lipopolysaccharide caused inflammation, but did not induce any relevant changes in ceruloplasmin (Dudek et al. 2013). Increased production of organic hydroperoxides was also found in relation to increased levels of stress hormones using either the d-ROMs (Costantini et al. 2008; Haussmann et al. 2012) or other assays (Sato et al. 2010; Balkaya et al. 2011).

Some authors used the d-ROMs assay to estimate production of free radicals (Al-Johany et al. 2009; Noguera et al. 2011). Organic hydroperoxides are likely to better reflect the basal free radical production than endproducts of lipid peroxidation (e.g., malondialdehyde). This is because organic hydroperoxides are generated earlier in the oxidative cascade than endproducts. However, organic hydroperoxides are primary oxidative damage molecules and none studies have tested the correlation between organic hydroperoxides and amount of free radicals produced. The correlation between free radical generation and organic hydroperoxides is unlikely to be very strong because there are various mechanisms and molecules that either prevent oxidation of biomolecules or remove organic hydroperoxides from the organism. It is therefore premature to infer about the production of free radicals from the results of the d-ROMs assay.

It has also been suggested that the d-ROMs assay is a measure of potential damage (Stier et al. 2015). This sentence implies that the d-ROMs test detects molecules that may potentially cause damage. This may certainly happen because molecules like organic hydroperoxides can be cleaved into free radicals, so fuelling the oxidative cascade.
However, it is unknown the extent to which this can happen (e.g., peroxidases reduce organic hydroperoxides to their corresponding alcohols before they can be cleaved). It therefore appears more correct to refer to oxidative damage because this is what the main molecules detected by the d-ROMs assay are. Note that, although peroxidases interact with organic hydroperoxides in the organism, it is unlikely that peroxidases cause significant interference with the d-ROMs test because of the low activity of peroxidases in plasma and the weak correlation between peroxidase activity and d-ROMs values (Costantini et al. 2011, 2012a).

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

Although the application of the d-ROMs test has been originally assessed for blood, recent studies showed that the d-ROMs test might also be applied to other biological matrices. For example, Castellini et al. (2003) found that d-ROMs values of seminal plasma of rabbits are correlated to those measured in blood plasma. Ito et al. (2009) found that plasma d-ROMs values were positively and significantly correlated ($r = 0.50$) with urine d-ROMs values. In another study, the d-ROMs test was applied to immune cells. Specifically, bovine peripheral blood mononuclear cells (component of the immune system) had been isolated from the whole blood and were then incubated for 2 and 7 days at different concentrations of mycotoxins to stimulate their activity (Bernabucci et al. 2011). Compared with the control, an increase of intracellular d-ROMs values (and also of malondialdehyde, which is an endproduct of lipid peroxidation) was observed, indicating the high sensitivity of the d-ROMs assay to perturbations of the oxidative balance caused by an immune response.
Similarly, in vitro induction of oxidative stress in prostate cancer cells resulted in the increase of d-ROMs values in the extracellular compartment, and of reactive oxygen species and DNA damage in the intracellular compartment (Tomasetti et al. 2010).

Finally, Criscuolo et al. (2010) found that the d-ROMs test may also be applied to haemolymph of invertebrates. Criscuolo et al. (2010) found that the d-ROMs values were higher in the haemolymph of short-lived male tarantulas (which also had higher superoxide production and lower antioxidant defences) than in the haemolymph of their long-lived females (which also had lower superoxide production and higher antioxidant defences).

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

Medical and veterinary research showed that the d-ROMs test has a significant diagnostic value of many pathological statuses, in agreement with other biomarkers of oxidative damage, such as the gold standard “isoprostanes” (e.g., correlation between d-ROMs values and isoprostanes = 0.68 in Lubrano et al. 2002). For example, d-ROMs values were found to be significantly associated with mortality in humans independently from established risk factors, including inflammation (Schöttker et al. 2015a).

It is now increasingly recognised that the d-ROMs is also a valuable test for the investigation of oxidative stress in ecological studies. Work has, for example, shown that d-ROMs values are associated with behavioural or fitness-related traits or reflect potential physiological costs induced by short-term activation of immune response or changes in the hormonal profile (Table 1). For example, experimental increase of plasma d-ROMs values was found to delay egg laying and reduce clutch size in a songbird (Costantini et al. 2016), which are two important fitness-related traits under female control. High plasma d-ROMs
values were found to be associated with reduced survival perspectives in two seabird species (Costantini et al. 2015; Herborn et al. 2016). Geiger et al. (2012) and Hau et al. (2015) also found that high plasma d-ROMs values were associated with shorter telomeres, which is an emerging marker of disease risk and biological ageing. Finally, a number of studies found that the d-ROMs test may inform about environmental quality (e.g., contamination level in Bonisoli-Alquati et al. 2010; food availability in van de Crommenacker et al. 2011a), adaptation to urbanisation (Lucas and French 2012; Costantini et al. 2014b) or variation in behavioural phenotype (Herborn et al. 2011; Costantini et al. 2012b).

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

A few authors raised critics to the d-ROMs assay, claiming that it is not a valid assay for the quantification of plasma oxidative status, organic hydroperoxides in particular. However, these critics have been based on serious methodological inadequacy and, importantly, on lack of respect of the protocols that have previously been validated.

Early criticisms about the lack of specificity were based on several mistakes done in the application of the d-ROMs assay (Erel 2005; Harma et al. 2006). For example, Erel (2005) used chelants for the preparation of samples, which interfere with the d-ROMs reaction (Banfi et al. 2006). Chelants sequester iron ions, which are needed for the reaction of the d-ROMs test and this was already explained in the validation study (Alberti et al. 2000). Hence, anticoagulants like EDTA or citrate should not be used.

Recently, similar critics to those of Harma et al. (2006) were surprisingly raised by Kilk et al. (2014). Again, these critics have been based on methodological inadequacy in
the application of the d-ROMs assay. First, Kilk et al. (2014) tested the capacity of the d-ROMs assay to assess oxidative damage using solutions of hydrogen peroxide. They found that the d-ROMs was able to weakly detect hydrogen peroxide only when it occurred at very high concentrations (≥ 50 µM) that are sometimes found in the plasma of individuals under severe oxidative stress. Hence, the authors concluded that the amount of peroxides in the plasma is a bit above the detection limit of the d-ROMs assay only under conditions of strong oxidative stress. The small capacity of the d-ROMs to measure hydrogen peroxide is, however, not surprising nor is it novel (see Liang et al. 2012) because the d-ROMs mainly measures organic hydroperoxides (e.g., Alberti et al. 2000; Liang et al. 2012; Colombini et al. 2016). The d-ROMs can detect organic hydroperoxides at concentrations well below 10 µM (Fig. 5), which have been found in the plasma of several vertebrates (e.g., Miyazawa 1989; Gerardi et al. 2002; Montgomery et al. 2011, 2012). For example, Gerardi et al. (2002) found a significant positive correlation between d-ROMs values and lipid hydroperoxides measured with the FOX2 assay.

Second, Kilk et al. (2014) found that d-ROMs readings of solutions containing ceruloplasmin and of sera decreased by decreasing the incubation temperature from 37°C (protocol of d-ROMs) to 23°C and to 4°C, while those of solutions containing hydrogen peroxide were less dependent on temperature. These results are again unreliable because (i) the d-ROMs assay is poorly sensitive to hydrogen peroxide, (ii) the Fenton reaction is highly dependent on temperature (e.g., Neyens and Baeyens 2003; Lee and Yoon 2004; Hussain et al. 2014) as also shown by the decrease of the readings of pure solutions of cumene hydroperoxide with the decrease of incubation temperature (Table 3), and (iii) the decrease of absorbance of pure solutions of cumene hydroperoxide with temperature is
similar to that of plasma samples (Table 3; note that the pure solution of organic hydroperoxides has to have an absorbance at 37°C similar to that of plasma samples in order to avoid “the regression to the mean effect”, i.e., when the change in absorbance is dependent on the initial absorbance), while that of ceruloplasmin shown in Kilk et al. (2014) is not.

Third, the authors speculated that thiols like albumin and glutathione might interfere with the assay. However, the amount of either albumin or glutathione that Kilk et al. (2014) added to the serum samples was pharmacological, while, as stressed by the same authors, glutathione did not affect the d-ROMs values at normal serum values.

Fourth, given this apparent temperature effect, the authors stated that they carried out the incubation at 23°C. Although the early assessment of the d-ROMs performance was carried out at room temperature (Alberti et al. 2000), the decrease of Fenton reaction with incubation temperature shown in Table 3 suggests that several of the conclusions made by the authors should be taken cautiously.

Fifth, the supposed correlation between d-ROMs values and activity of ceruloplasmin shown in figure 7 is not reliable because (i) it is an artifact of plotting two species having different levels of both d-ROMs values and ceruloplasmin and (ii) more importantly because the values of absorbance of sera reported in figure 7 are not compatible with the normal absorbance values of the assay (see e.g. values of absorbance in Table 2 for a volume of 200 μl within a plate well).

**Improvements in the application of the d-ROMs assay**
The d-ROMs assay can be performed using either cuvettes or well plates. The use of well plates may face the experimenter with an issue when there is any formation of precipitate on the bottom of the well. To overcome this problem, incubation can be done in tubes and, straightaway the end of incubation, tubes can be centrifuged and the supernatant used for the readings. Another problem may be with the plasma colour when this is very yellow. In this case, it is important to increase the wavelength at which readings are taken (e.g., 505 nm or even more), considering that, for example, one peak of absorbance of lutein is at ≈ 476 nm. As with the plasma colour, it should also be paid attention to whether plasma samples look red because of haemolysis. Iamele et al. (2002) found that a concentration of haemoglobin above 0.068 mM in human serum may interfere with the d-ROMs measurements.

The d-ROMs assay may not be sensitive, especially for young individuals, whose d-ROMs values may be low (Costantini et al. 2006). In order to improve the performance of the assay, it can be used more plasma than usual, but carefulness is needed in order to avoid any interference of plasma colour or alterations of the pH of the buffer.

Finally, it has been suggested that it might be interesting to measure fatty acids and/or cholesterol in plasma and to express d-ROMs values also per unit of fatty acids (Pérez-Rodríguez et al. 2015). This is because fatty acids and cholesterol are substrates for formation of organic hydroperoxides. Note that caution is needed because d-ROMs values do not only refer to lipid hydroperoxides but also to those formed from oxidation of other substrates and the correlations between d-ROMs values and either fatty acids or cholesterol are often low (e.g., Casagrande et al. 2011; Kotani et al. 2013). It might, however, be
interesting to assess if, together with uncorrected values, d-ROMs values corrected for the number of substrates that occur in plasma also provide valuable biological information.

Conclusions
Conversely to many colorimetric assays, the d-ROMs test has been properly assessed using electron paramagnetic resonance. All the direct and indirect evidence shows that the d-ROMs test is a valuable assay for the quantification of the plasma or serum oxidative status (higher values indicating higher oxidative damage), due to primary oxidation products of biomolecules (mainly organic hydroperoxides, but also endoperoxides and organic chloramines). Moreover, values of d-ROMs show significant individual repeatability over time (e.g., Costantini et al. 2007; Hau et al. 2015; Herborn et al. 2016). Experimental data also show that this assay is very sensitive to specific pro-oxidant agents and can provide ecologically valuable information, suggesting that this test might serve as a blood-derived biomarker to assess the impact of oxidative damage on health and fitness perspectives in animals. Clearly, as for any other metric of oxidative damage, the d-ROMs test should also be used in combination with other assays in order to better assess the individual oxidative balance. It is recommended that ecologists, interested in pursuing research in oxidative stress ecology, get sufficient basic background in biochemistry before making their own choice about what assay is suitable for their work.

Acknowledgments
I thank the Associate Editor, three anonymous reviewers, Angelo Alberti and Stefania Casagrande for providing valuables comments that helped me to improve the presentation
of the article; Katherine Herborn for kindly providing data on Phalacorcorax aristotelis reported in Table 3. Research Foundation of Flanders has supported this work.

References


Casagrande S, Pinxten R, Zaid E, Eens M (2014) Carotenoids, birdsong and oxidative status: administration of dietary lutein is associated with an increase in song rate and circulating antioxidants (albumin and cholesterol) and a decrease in oxidative damage. PLoS ONE 9:e115899


Lee C, Yoon J (2004) Temperature dependence of hydroxyl radical formation in the $hv/Fe^{3+}/H_2O_2$ and $Fe^{3+}/H_2O_2$ systems. Chemosphere 56:923-934

6


Lumeij JT, de Bruijne JJ (1985) Blood chemistry reference values in racing pigeons (*Columba livia domestica*). Avian Pathol 14:401-408


van de Crommenacker J, Komdeur J, Richardson DS (2011b) Assessing the cost of helping: the roles of body condition and oxidative balance in the Seychelles warbler (*Acrocephalus sechellensis*). PLoS ONE 6:e26423


Table 1 Examples of how changes of d-ROMs values in relation to a number of behavioural traits or stressors have been found across a broad variety of organisms

<table>
<thead>
<tr>
<th>Trait or stressor</th>
<th>Taxon</th>
<th>Effect</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominance status during the breeding season</td>
<td><em>Acrocephalus sechellensis</em></td>
<td>Dominant males had higher d-ROMs values</td>
<td>van de Crommenacker et al. 2011b</td>
</tr>
<tr>
<td>Dominance status during the breeding season</td>
<td><em>Mandrillus sphinx</em></td>
<td>High-ranking males had higher d-ROMs values</td>
<td>Beaulieu et al. 2014</td>
</tr>
<tr>
<td>Food habits</td>
<td>33 bat species</td>
<td>Species with a frugivorous diet had the lowest d-ROMs values, followed by omnivorous and animalivorous species</td>
<td>Schneeberger et al. 2014</td>
</tr>
<tr>
<td>Food habits</td>
<td><em>Pygoscelis papua</em></td>
<td>Colonies with the highest δ13C and δ15N values had the highest d-ROMs values</td>
<td>Beaulieu et al. 2015</td>
</tr>
<tr>
<td>Heat stress</td>
<td><em>Taeniopygia guttata</em></td>
<td>Increase of d-ROMs values</td>
<td>Costantini et al. 2012a</td>
</tr>
<tr>
<td>Immune response</td>
<td><em>Falco tinnunculus</em></td>
<td>Increase of d-ROMs values</td>
<td>Costantini and Dell’Omo 2006</td>
</tr>
<tr>
<td>Immune response</td>
<td><em>Carollia perspicillata</em></td>
<td>Increase of d-ROMs values</td>
<td>Schneeberger et al. 2013</td>
</tr>
<tr>
<td>Malaria infection</td>
<td><em>Parus major</em></td>
<td>Increase of d-ROMs values</td>
<td>Isaksson et al. 2013</td>
</tr>
<tr>
<td>Solicitation display</td>
<td><em>Hirundo rustica</em></td>
<td>Begging bout duration was negatively predicted by d-ROMs values but only after food deprivation.</td>
<td>Boncoraglio et al. 2012</td>
</tr>
<tr>
<td>Song behaviour</td>
<td><em>Sturnus vulgaris</em></td>
<td>Decrease of song rate with increase of d-ROMs values</td>
<td>Casagrande et al. 2014</td>
</tr>
</tbody>
</table>
Table 2 Comparison of absorbance values between samples that were either not treated or treated with sodium azide (inhibitor of ceruloplasmin activity). Affinity of sodium azide for ceruloplasmin (and so its capacity to inhibit ceruloplasmin activity) varies across species, but a concentration of 1 mM of sodium azide in a buffer with a 7.4 pH has been shown to reduce ceruloplasmin activity in both humans and chickens (Musci et al. 1999). The capacity of sodium azide to inhibit ceruloplasmin was also suggested to increase with the decrease of pH (Musci et al. 1999). This is important because the buffer used for the d-ROMs assay has a pH of 4.8, hence much lower than that used in Musci et al. (1999). Data on *Phalacrocorax aristotelis* are from Herborn et al. 2016. Values are expressed as mean ± standard deviation. Coefficient of variation refers to variation in absorbance values between samples that were either not treated or treated with sodium azide. Difference in absorbance value mean between samples that were either not treated or treated with sodium azide is also shown as percentage.

<table>
<thead>
<tr>
<th>Species</th>
<th>No sodium azide</th>
<th>1 mM sodium azide</th>
<th>Coefficient of variation</th>
<th>Difference expressed as %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Serinus canaria</em></td>
<td>0.132±0.015</td>
<td>0.135±0.017</td>
<td>3.03±1.75</td>
<td>+3.68</td>
</tr>
<tr>
<td><em>Sturnus vulgaris</em></td>
<td>0.158±0.021</td>
<td>0.163±0.013</td>
<td>4.81±1.34</td>
<td>+2.55</td>
</tr>
<tr>
<td><em>Taeniopygia guttata</em></td>
<td>0.182±0.053</td>
<td>0.186±0.052</td>
<td>2.20±1.30</td>
<td>+1.88</td>
</tr>
<tr>
<td><em>Calonectris diomedea</em></td>
<td>0.110±0.009</td>
<td>0.114±0.009</td>
<td>2.66±2.50</td>
<td>+3.95</td>
</tr>
<tr>
<td><em>Phalacrocorax</em></td>
<td>0.183±0.025</td>
<td>0.175±0.019</td>
<td>4.90±2.50</td>
<td>-4.37</td>
</tr>
<tr>
<td>aristotelis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>758</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>759</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3 Percentage of decrease of absorbance at 24 and 4°C as compared to 37°C, which is the temperature required by the d-ROMs protocol. Note that the among sample variation is to be expected given that in plasma samples there are several groups of organic hydroperoxides that contribute to the reading and these differ in concentration among individuals. Moreover, in plasma samples there are other primary oxidative damage molecules that contribute to the reading (e.g., organic chloramines that derive from oxidation of proteic amine groups; endoperoxides; Alberti et al. 2000; Liang et al. 2012)

<table>
<thead>
<tr>
<th></th>
<th>% of decrease of absorbance at 24°C as compared to 37°C</th>
<th>% of decrease of absorbance at 4°C as compared to 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumene hydroperoxide 1</td>
<td>39</td>
<td>62</td>
</tr>
<tr>
<td>Cumene hydroperoxide 2</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>Standard 1</td>
<td>46</td>
<td>71</td>
</tr>
<tr>
<td>Standard 2</td>
<td>50</td>
<td>69</td>
</tr>
<tr>
<td>Plasma 1</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>34</td>
<td>54</td>
</tr>
<tr>
<td>Plasma 3</td>
<td>36</td>
<td>57</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>38</td>
<td>56</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>12.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>
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; 1O2 = 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
Figure 1
Figure 2

A. \[ \text{reaction} \]

B. \[ \text{chemical structures} \]

Val-OOH  Lys-OOH  Leu-OOH
Figure 3
Figure 4
Figure 5

Cumene hydroperoxide (µM)

Absorbance at 505 nm