Comparative Biochemistry and Physiology, Part A 298 (2024) 111750

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Haemolysis overestimates plasma oxidative stress biomarkers in free-ranging roe deer

Amandine Herrada [a,*], Pauline Vuarin [a], François Débias [a], Alexia Gache [a], Philippe Veber [a], Maryline Pellerin [b], Louise Cheynel [c], Jean-François Lemaître [a], Emmanuelle Gilot-Fromont [a,d], Benjamin Rey [a]

[a] Université Lyon 1, UMR CNRS 5558, Laboratoire de Biométrie et Biologie Evolutive, 69622 Villeurbanne, France
[b] Office Français de la Biodiversité, Direction de la Recherche et de l'Appui Scientifique, Châteauvillain-Arc-en-Barrois, France
[c] Université Lyon 1, UMR CNRS 5023, Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, 69622 Villeurbanne, France
[d] Université de Lyon, VetAgro Sup - Campus Vétérinaire de Lyon, Marcy-L 'Etoile, France

ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Field studies
Haemoglobin
Haemolysis
Oxidative stress markers
Red blood cell
Roe deer

ABSTRACT

Quantifying oxidative stress has garnered extensive interest in evolutionary ecology and physiology since proposed as a mediator of life histories. However, while the theoretical framework of oxidative stress ecology is well-supported by laboratory-based studies, results obtained in wild populations on oxidative damage and antioxidant biomarkers have shown inconsistent trends. We propose that red blood cell lysis could be a source of bias affecting measurements of oxidative stress biomarkers, distorting the conclusions drawn from them. Using an experimental approach consisting of enriching plasma from roe deer with lysed red blood cells, we show that the values of commonly used oxidative stress biomarkers linearly increase with the degree of haemolysis – assayed by haemoglobin concentration. This result concerns oxidized proteins (carbonyls) and lipids (TBARS), as well as enzymatic (superoxide dismutase) and non-enzymatic (trolox assay, OXY assay) antioxidant markers. Based on 707 roe deer blood samples collected in the field, we next show that the occurrence of haemolysis in plasma samples is negatively related to age. Finally, we illustrate that considering the variance explained by age-related haemolysis improves explanatory models for inter-individual variability in plasma oxidative stress biomarkers, without substantially altering the estimates of the parameters studied here. Our results raise the question of the veracity of the conclusions if the degree of haemolysis in plasma is not considered in animal models such as roe deer, for which the occurrence and severity of haemolysis vary according to individual characteristics. We recommend measuring and controlling for the degree of haemolysis be considered in future studies that investigate the causes and consequences of oxidative stress in ecophysiological studies.

1. Introduction

Since the first evidence that oxidative stress may underpin life history trade-offs in the early 2000s (Alonso-Alvarez et al., 2004; Wiersma et al., 2004), the quantification of oxidative stress markers has seen an unprecedented surge in ecophysiological studies (Costantini et al., 2014; McGraw et al., 2010). Oxidative stress refers to the detrimental accumulation of oxidized molecules in cells and tissues, which may, impair organ function, promote age-related degenerative diseases and accelerate the ageing process (Finkel and Holbrook, 2000). Oxidative stress typically occurs when the production rate of reactive oxygen species (ROS) by metabolic or immune enzymes overcomes i) the preventive actions of antioxidant and radical scavenger molecules and ii) the curative mechanisms of repair, removal and renewal of oxidized molecules (Burton and Jauniaux, 2011; Halliwell and Gutteridge, 2015).

The oxidative stress life-history theory (*sensu* Monaghan et al., 2009) posits that allocation towards energy-demanding traits or functions (*e.g.* growth, reproduction) would stimulate the generation of ROS from aerobic metabolism and/or reduce the capacity to allocate in antioxidant defences and repair machinery, to the detriment of redox homeostasis (Costantini, 2008; Dowling and Simmons, 2009). Accordingly, oxidative stress would represent a net somatic cost of the physiological processes underpinning growth and reproduction (Monaghan et al., 2009), and would provide physiological support to the widely

* Corresponding author.
E-mail address: amandine.herrada@univ-lyon1.fr (A. Herrada).

https://doi.org/10.1016/j.cbpa.2024.111750
Received 26 July 2024; Received in revised form 20 September 2024; Accepted 20 September 2024
Available online 21 September 2024

documented life history trade-offs (Zera and Harshman, 2001). However, decades after the seminal papers, hundreds of studies, performed under both laboratory and natural conditions and on a variety of animal models, bring mixed support for oxidative stress life-history theory (Alonso-Alvarez et al., 2017; Christensen et al., 2015; Speakman and Garratt, 2014). For instance, support to the idea that reproduction inflicts an oxidative cost is far from unanimous (Metcalfe and Monaghan, 2003), as oxidative status was found to be uncoupled from reproductive effort in numerous studies, either in free-ranging (Nussey et al., 2009) or captive animals (Aloise King et al., 2013; Ołdakowski et al., 2015). Similarly, the idea that fast growth should favour oxidative stress is not universally accepted (but see Smith et al., 2016; Costantini, 2022 for reviews) and the magnitude of the effect of oxidative stress on the ageing process is also subject to debates (Selman et al., 2012; Speakman and Garratt, 2014).

This lack of consensus in the literature towards the idea that oxidative stress plays a key role in mediating life-history trade-offs may have several origins (Speakman et al., 2015). It has been proposed that the role of oxidative stress in life history evolution is conceptually flawed and that accurate experimental tests of the theory would be much more complex than generally performed (Speakman and Garratt, 2014). Hence, several theories have emerged in an attempt to explain why allocation in a life history trait does not necessarily result in a measurable oxidative cost (for example in the case of reproduction, the *oxidative shield* hypothesis (Blount et al., 2016), the *hormesis during reproduction* hypothesis (Costantini et al., 2014), or the *extortion for reproduction* hypothesis (Alonso-Alvarez et al., 2017)).

Alternatively, there is increasing evidence that the lack of general agreement around the oxidative stress life-history theory may be due to methodological issues (Hõrak and Cohen, 2010; Metcalfe and Monaghan, 2013; Speakman et al., 2015) raising the question of whether oxidative stress is accurately quantified (Speakman and Garratt, 2014). For technical and logistical reasons, research into the causes and consequences of oxidative stress in wildlife focuses almost exclusively on markers of oxidative damage on one hand, and certain molecules indicative of the antioxidant machinery on the other hand. It rarely embraces the complexity of oxidative stress regulation, ignoring the multiple sources of pro-oxidant molecules, and the wide range of damaged molecules repair and removing pathways (but see Rey et al., 2016). Moreover, among the commonly used markers, some are poorly correlated (Christensen et al., 2015), which poses problems of interpretation and makes comparisons between studies difficult. In fact, there is a wide range of markers used to assess oxidative stress. These include markers for oxidative damage to lipids, proteins, or DNA, as well as markers that evaluate enzymatic and non-enzymatic antioxidant defences, which can be either specific or non-specific (Czerska et al., 2015). To add to the challenge, the majority of field studies rely on markers measured in blood. This raises the issue about the biological representativeness of markers measured in this tissue, when the effects of allocation to a given trait or function on oxidative stress are known to be tissue-specific (*e.g.* Garratt et al., 2012; Ołdakowski and Taylor, 2018). Blood can be collected in a minimally invasive manner, enabling repeated sampling in the animals being monitored (Milner et al., 2003; Norte et al., 2008), and thus representing a considerable asset for longitudinal studies (Sheldon et al., 2022). Nevertheless, blood is also a complex and versatile tissue, whose composition may be subjected to changes in response to external factors once collected (*e.g.* storage conditions, Pawlik-Sobecka et al., 2020; Vogelaar et al., 2002). Blood is generally centrifuged after collection, and oxidative stress markers are typically measured in the plasma. Such measurements require precautions and several articles have pointed out methodological recommendations and limitations (*e.g.* Cohen et al., 2007; Costantini, 2011). Among them, it has been raised that haemolysis - the rupturing of red blood cell (RBC, or erythrocytes) and the release of their content into the surrounding fluid - could affect plasma composition and/or interfere with the assays (Fraser et al., 2011; Heyer et al., 2012; Selman et al., 2012). Assay protocols and guidelines for the determination of oxidative stress markers often mention that haemolysed plasma samples should not be used due to potential interferences with assay reagents (*e.g.* Levine et al., 1990; Dalle-Donne et al., 2003). However, studies that formally test the extent to which the degree of cell lysis affects measures of oxidative stress markers are rare and limited to human studies (Dreißigacker et al., 2010), even though proofs have been reported that haemolysis may introduce a significant bias to most clinical tests (*e.g.* Lippi et al., 2011a, 2011b; Delianu et al., 2020). There are also no clear recommendations in the literature regarding the approach to adopt when plasma is partially haemolysed, nor any information on the degree of haemolysis at which a sample, irrespective of its intrinsic value or collection effort, should be considered inappropriate and thus discarded. Furthermore, the strength of the relationship between haemolysis and oxidative stress biomarkers still remains unknown, as well as whether this relationship is consistent across different types of biomarkers (*i.e.* between damage and defence biomarkers, but also among biomarkers within the same category).

Haemolysis may have two major origins. The *in vivo* haemolysis results from the death of senescent or damaged RBCs within the circulation, a process that may be exacerbated by high intensity exercise and by several physio-pathological conditions. In most cases however, haemolysis occurs outside the body during blood sampling process, handling, transport or storage conditions, or during centrifugation, and is referred as *in vitro* haemolysis (Heireman et al., 2017; Lippi et al., 2011b; Tomin et al., 2021). Although haemolysis occurs after blood collection in such cases, a large body of biomedical and laboratory animal literature tells us that the occurrence of *in vitro* haemolysis in sampled blood is related to inter-individual variations in the intrinsic susceptibility of RBCs to mechanical, oxidative and osmotic trauma. In humans, sex, age, health status, body condition and ethnicity of individuals contribute to explain such inter-individual variability (Heireman et al., 2017; Shopsowitz and Shih, 2021).

While considerable efforts in evolutionary biology studies have focused on determining the oxidative status, it seems important to check whether patterns of oxidative stress markers could be affected by factors modulating the susceptibility of RBCs to haemolysis. In other words, we must discern whether the effects of such factors on oxidative stress markers truly reflect oxidative stress patterns or are merely influenced by haemolysis.

To this end, we collected plasma from wild roe deer as part of a long-term population monitoring program to meet three sub-objectives: i) based on an *in vitro* approach consisting in enriching plasma with increasing amount of disrupted RBCs, we first assessed the extent to which haemolysis affects measurements of common plasma markers of oxidative damages and antioxidant defences, ii) we then measured haemoglobin concentrations on plasma collected on roe deer from two populations and investigated whether the occurrence of haemolysis is influenced by individual (sex, body mass, age) or environmental (environment of low quality *versus* high quality food resources) factors, and iii) finally, we examined the extent to which ignoring the degree of haemolysis may distort our understanding of the individual and/or environmental factors that govern patterns of oxidative stress markers.

2. Materials and methods

2.1. Study populations

All data and samples come from two roe deer populations (*Capreolus capreolus* (Linnaeus, 1758)) located in closed forests managed by the French Agency for Biodiversity (OFB). The Trois-Fontaines forest (TF – 1360 ha), located in north-eastern France (48°43′N, 4°55′E), is characterized by a continental climate with rich soils, providing a high-quality habitat for roe deer. The Chizé forest (CH – 2614 ha), located in western France (46°05′N, 0°25′W), is characterized by a temperate oceanic climate with frequent summer droughts and poor soils, offering

therefore a lower quality habitat for roe deer (Pettorelli et al., 2006). These differences between populations have repercussions notably on body mass, immunity and survival (body mass: Douhard et al., 2013, immunity: Gilot-Fromont et al., 2012, survival: Gaillard et al., 1993).

The long-term capture-mark-recapture program that has been conducted in the two populations for over 45 years offers a unique opportunity to monitor roe deer throughout their lives, giving access to precious data on individual (*e.g.* body mass, sex, age) and environmental characteristics (Gaillard et al., 1993). Every winter (December to March), roe deer are netted and transported in wooden crates to a handling area. Once a roe deer is captured, its sex and body mass (to the nearest 0.1 kg) are recorded, and a basic clinical examination is performed. All individuals included in our analyses were of known age because they were either caught as new-borns in spring or as *ca.* 8 months old during winter captures, when they still have their milk teeth (Flerov, 1952). Individuals are equipped with individually recognizable ear-tags and collars (either numbered, VHF or GPS collars), get a unique id, and are closely monitored throughout their lifetime. Since 2010, blood samples are collected (1 ml/kg with a maximum of 20 ml) from the jugular vein in 20 ml syringes attached with sterile hypodermic needles (gauge 19, 1.1 × 38 mm). Blood is immediately transferred to heparinized collection tubes (4 ml Vacutainer, BD Medical, France) and spun at 2000 *g* for 10 min. Plasma samples are then transferred to cryogenic storage tubes and frozen on site at −80 °C in a portable freezer (Telstar SF 8025) until laboratory analyses. Red blood cell pellets are then rinsed with PBS (1 *v*/v) before re-centrifugation. The buffy coat containing white blood cells is removed and the pellets containing RBC are then frozen (and thus lysed) under the same conditions. At the end of the procedure, roe deer are released at their site of capture. All previous procedures were approved by the Ethics Committee of the University Lyon 1, France (project DR2014–09, June 5, 2014).

2.2. *In vitro* constitution of a range of haemolysed plasma

We used visually non-haemolysed plasma from 10 randomly selected individuals, to constitute a range of low- to highly-haemolysed plasma by sequentially enriching the plasma with RBCs from the corresponding blood samples. For each individual, the most haemolysed plasma was obtained by mixing one volume (15 µl) of RBCs with 30 volumes (465 µl) of the corresponding plasma. The solution was then diluted six times with plasma following a serial dilution procedure (*i.e.* 240 µl of haemolysed plasma with 240 µl of original plasma at each dilution). We therefore ended up with 7 plasma samples gradually concentrated in RBCs. An eighth sample consisting of the original plasma used for dilution, without any addition of RBCs, constituted the least haemolysed plasma sample. In total, we obtained 80 plasma samples from 10 different individuals, with 8 degrees of haemolysis per individual.

The precise degree of haemolysis was further assessed by measuring the concentration of haemoglobin (see below). In each of the samples, we also quantified oxidative damage and antioxidant markers (see below).

Note that the quantity of plasma available was insufficient to measure all markers on each sample. The experiment was thus carried out twice, still using samples from the Trois-Fontaines population: the first time with 10 samples collected in 2022, and the second time with 10 samples collected in 2023 from different individuals. Activity of superoxide dismutase (SOD) and total antioxidant measurements (see below) were carried out on samples collected in 2023, while all other markers were measured in plasma collected in 2022 (see below). In both cases, haemoglobin was measured the same way.

2.3. Blood sampling in roe deer populations

We used 707 plasma samples, corresponding to 465 individuals, in both populations between 2016 and 2019 to explore individual (sex, age, body mass) and environmental (CH *vs.* TF study site) factors explaining haemolysis patterns in roe deer plasma. Of these individuals, 88 had plasma collected in two different years, 59 in three and 12 in four. On these samples, we measured haemoglobin concentrations, as well as oxidative damage and antioxidant markers as described below, with the exception of the total antioxidant capacity (TAC) assay.

2.4. Oxidative damage and antioxidant assays

We measured three complementary plasma biomarkers of oxidative damages to molecules (d-ROMs, TBARS, protein carbonyl content) and three biomarkers of antioxidant defences (OXY-test, TAC, SOD activity). The choice of markers was carefully considered so as to be representative of those commonly used in the literature. These markers cover molecules of very different nature (*e.g.* damages measured on lipids *vs.* proteins) or of different specificity (*e.g.* highly specific antioxidant such as SOD *vs.* non-specific antioxidant such as TAC). All markers were assayed in accordance with the manufacturer's instructions.

2.4.1. d-ROMs test (Diacron International)

The d-ROM test allows measuring the total amount of reactive oxygen metabolites (ROMs), mainly hydroperoxides. ROMs are capable of generating alkoxyl and peroxyl radicals following the Fenton's reaction in the presence of iron under acidic conditions. These radicals react with the colourless alkyl-substituted aromatic amine probe to give a pink coloration which can be quantified photometrically at 540 nm (SAFAS MP96 microplate reader, Monaco). The concentrations of ROMs were assayed in duplicate against standard curves and expressed as mg H_2O_2. dl^{-1}. Average intra-plate coefficient of variation (CV) calculated from the duplicates was less than 6 %.

2.4.2. Thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemical, reference number: 10009055)

Malondialdehyde (MDA) is one of the major end-products of polyunsaturated fatty acid peroxidation and is commonly used as an index of oxidative damages to lipids. MDA can be measured as the MDA-TBA (Thiobarbituric acid) adduct formed by the reaction of MDA and TBA under high temperature (95 °C), following reading at 540 nm with a spectrophotometer.

The plasma levels of TBARS (expressed as MDA concentrations) were determined against a standard curve of MDA, and are expressed in µM MDA. Average intra-plate coefficient of variation was less than 4 %.

2.4.3. Protein carbonyl content assay Kit (Sigma-Aldrich, reference number: MAK094)

The oxidation of protein side chains (mainly of proline, arginine, lysine and tyrosine) results in the accumulation of stable carbonyl groups that can be used as a measure of oxidative damages to proteins. In this assay, carbonyl groups are being derivatized with 2,4-dinitrophenyl-hydrazine (DNPH) and forms stable dinitrophenyl (DNP) hydrazone adducts (aldehydic and ketonic). This molecule can then be spectrophotometrically detected at 380 nm and is proportional to the carbonyl groups content of a sample. Circulating levels of carbonyl groups are then normalized by the amount of total proteins present in the sample, which are quantified using the BCA (Bicinchoninic acid) protein assay (Sigma-Aldrich; Catalog Number 71285-M) using bovine serum albumin as standard. Carbonyl levels are then expressed in nmol carbonyl.mg^{-1} protein. Average intra-plate coefficient of variation was less than 10 %.

2.4.4. OXY-Adsorbent test (Diacron International)

OXY-adsorbent test allows assaying the total (non-specific) antioxidant capacity of plasma to *in vitro* oxidation initiated by adding a known amount of hypochlorous acid (HClO). The remaining HClO that escapes the antioxidant system oxidizes an added alkyl-substituted aromatic amine and creates a pink-coloured derivative that can be read spectrophotometrically at 540 nm. The assay was carried out in duplicate on

plasma diluted 1/100 according to recommendation. The antioxidant defences can then be quantified as inversely proportional to absorbance and read against a standard curve. Levels are expressed in μmol HClO neutralized.ml^{-1}. Average intra-plate coefficient of variation was less than 6 %.

2.4.5. Total antioxidant capacity assay (Sigma-Aldrich, reference number: MAK187)

TAC is another measure of non-specific antioxidant capacity. This marker relies on the reduction of Cu^{2+} into Cu^{+} by small antioxidant molecules present in the plasma. The resulting Cu^{+} creates a coloured complex with a dye reagent which can be quantified spectrophotometrically at 570 nm, the colour intensity being proportional to the amount of antioxidant molecules in the sample. The plasma levels of antioxidants are determined in duplicate against a standard curve of Trolox, a water-soluble vitamin E analogue. Levels of antioxidant capacity are expressed in μM Trolox. Average intra-plate coefficient of variation was less than 3 %.

2.4.6. Superoxide dismutase activity determination (Sigma-Aldrich, reference number: CS0009)

Superoxide dismutase (SOD) is the first line of antioxidant defences that catalyses the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. Its activity can be quantified as the capacity of plasma to remove superoxide anions that are generated *in vitro* by the xanthine/xanthine oxidase (X/XO) system. These superoxide anions, along with a given water-soluble tetrazolium salt, form a water-soluble formazan dye that can be measured spectrophotometrically at 450 nm. The assay was carried out in duplicate on plasma diluted 1/5 to fit in the SOD standard curve. In accordance with guidelines, a first spectrophotometric reading was performed before adding the enzyme working solution. The resulting O.D. (Optical Density) values were subtracted from each well after the second reading so that each sample had its own blank. This procedure has been adopted to eliminate possible interference with coloured molecules in each well so as to control for the effects of haemolysis. SOD activity was expressed as an inhibition rate (%), *i.e.* the percent inhibition of the tetrazolium salt reduction, which represents the amount of SOD present in the sample. Average intra-plate coefficient of variation was less than 9 %.

2.5. Plasma haemoglobin concentration assay

Haemoglobin is the iron-containing oxygen-transport protein that is primarily found in RBCs. The haemoglobin assay kit (Sigma-Aldrich, reference number: MAK115) is based on the Triton/NaOH method, in which hemes are oxidized in alkaline media to form a colorimetric product that can be measured spectrophotometrically at 400 nm. Levels are assayed in duplicate, read against a standard curve and expressed in mg.dl^{-1}. Average intra-plate coefficient of variation was less than 10 %.

2.6. Statistical analyses

All statistical analyses were performed using R (version 4.2.1; R core Team, 2021).

2.6.1. Assessing the effect of haemolysis on oxidative damages and antioxidant biomarkers

We used linear mixed effects models (*lme4* package; Bates et al., 2015) to analyse the relationships between haemoglobin concentration and the different oxidative stress biomarkers in the range of *in vitro* haemolysed plasma samples (one model was performed for each biomarker). In each model, the biomarker was set as the response variable and haemoglobin concentration as a covariate, whereas individual identity was included as a random factor to account for multiple measurements on the same individuals. The normality and homoscedasticity of the residuals of the models were checked by visual inspection of their distribution (QQ-plot and standardized residuals plot, respectively). For each variable, we report its coefficient (β), as well as its 95 % confidence intervals (95 % CI), and marginal and conditional R^2 of the models.

2.6.2. Identifying the factors affecting haemolysis in roe deer populations

We used linear mixed effects models to identify individual (*i.e.* age and body mass as continuous variables, and sex) or environmental (low quality *versus* high quality food resources) factors that could explain patterns of haemoglobin concentrations in the plasma of individuals from both populations. A total of 364 plasma samples were collected in Chizé (199 females and 165 males) and 343 in Trois-Fontaines (176 females and 167 males). Haemoglobin concentration was set as the response variable. Explanatory variables were the fixed effects of individual or environmental characteristics. Individual identity and plate number for each haemoglobin measurement were set as random effects to take into account the non-independence between measurements. For juveniles ($\leq$ 1 year old), body mass was recalculated as their predicted mass on the 27th of January (*i.e.* the median date of roe deer captures in our dataset), since birth date is negatively associated to early body growth in roe deer (Douhard et al., 2017). To avoid collinearity issues between body mass and other individual variables, we calculated the residuals of a linear mixed effects model with body mass as the dependent variable, age and sex as the fixed effects, and individual identity as the random effect. Residuals were then used as a proxy of body mass accounting for the effects of age and sex. We used a model selection procedure to identify the fixed effects that best explained the variance in haemoglobin concentrations. Hence, it was implemented from the full model based on the Akaike Information Criteria corrected for small sample sizes (AICc), using the *dredge* function from the package MuMin (Bartoń, 2013). All models within 2 ΔAICc were retained (*i.e.* difference in AICc between a given model and the model with the lowest AICc; Burnham and Anderson, 2002), except those with uninformative parameters, *i.e.* parameters that do not bring meaningful information or explanatory power to the model's predictions (Arnold, 2010). Finally, between all models within 2 ΔAICc, we retained the simplest model (*i.e.* with the fewest parameters) to satisfy parsimony rules.

The normality and homoscedasticity of the residuals of the selected models were checked by visual inspection of their distribution (QQ-plot and standardized residuals plot, respectively). Haemoglobin concentrations were log-transformed when used as a response variable to meet these assumptions. For each variable, we report its coefficient (β), as well as its 95 % confidence intervals (95 % CI), and marginal and conditional R^2 of the models.

2.6.3. Influence of the level of haemolysis in the plasma on the assessment of redox status in roe deer populations

We used linear mixed effects models and performed similar analyses to those described above to test whether taking the degree of haemolysis into account could lead to different results when assessing the effects of individual and/or environmental factors on oxidative stress and antioxidant biomarkers. For this purpose, we have chosen malondialdehyde and the superoxide dismutase to illustrate the effects on oxidative damage and antioxidant markers, respectively, as they both show strong relationship with haemolysis (*i.e.* strong R^2, see Table 1).

We first analysed the relationships between the biomarkers (*i.e.* malondialdehyde or superoxide dismutase), individual (*i.e.* age, sex, body mass) and environmental (low quality *versus* high quality food resources) factors as previously described. We next ran the same models with haemoglobin concentration as an additional covariate to account for the proportion of variance explained by haemoglobin concentration in the plasma samples. Individual identity and sampling plate were also included as random effects, and the model selection procedure was similar to that described above. Malondialdehyde concentrations were log-transformed to meet the assumptions of normality and homoscedasticity in linear models.

Table 1
Parameter estimates of linear mixed effects models describing the relationship between makers of oxidative damages (dROMs, Carbonyls and MDA) or antioxidant defences (OXY, TAC assay, SOD activity) with haemoglobin concentration in roe deer plasma with varying degrees of haemolysis. Parameter estimates are given with 95 % confidence intervals (95 %CI). Models included individual identity and assay plate number as random effects.

	Biomarkers of oxidative damages		Biomarkers of antioxidant defences	
Variables	**dROMs**		**OXY**	
	Estimate	95 %CI	Estimate	95 %CI
Intercept	9.327	[8.520; 10.134]	242.638	[221.066; 264.211]
Haemoglobin	$-4.817e10^{-4}$	[−0.001; $2.653e10^{-5}$]	0.027	[0.007; 0.047]
Marginal R^2			0.029	
Conditional R^2			0.677	
	Carbonyls		**TAC**	
	Estimate	95 %CI	Estimate	95 %CI
Intercept	4.575	[3.102; 6.048]	147.751	[124.308; 171.194]
Haemoglobin	0.018	[0.017; 0.020]	0.825	[0.795; 0.855]
Marginal R^2	0.736		0.942	
Conditional R^2	0.892		0.975	
	MDA		**SOD**	
	Estimate	95 %CI	Estimate	95 %CI
Intercept	10.165	[2.760; 17.569]	4.752	[2.723; 6.780]
Haemoglobin	0.049	[0.035; 0.062]	0.061	[0.056; 0.066]
Marginal R^2	0.352		0.866	
Conditional R^2	0.541		0.890	

3. Results

3.1. Assessing the effect of haemolysis on oxidative damages and antioxidant biomarkers

The RBC enrichment protocol covered a spectrum of haemolysed plasma ranging from a haemoglobin concentration of 29.4 ± 14.5 mg/dl (lowest haemolysis, without RBC addition) to 766.6 ± 152.9 mg/dl (highest haemolysis).

The level of reactive oxygen metabolites (dROMs) was not significantly affected by the degree of haemolysis (Table 1; Fig. 1A). In contrast, measures of oxidized proteins (carbonyls) and lipid peroxidation (MDA) linearly increased with the degree of haemolysis in plasma samples (Table 1; Fig. 1B,C; marginal R^2 = 0.736 and 0.352 for carbonyls and MDA, respectively).

Among antioxidant markers, SOD activity and TAC increased drastically and linearly with the degree of haemolysis (Table 1; Fig. 1E,F; marginal R^2 = 0.866 and 0.942 respectively for SOD and TAC). Although less pronounced, the non-specific OXY antioxidant marker was also positively correlated with the degree of haemolysis (Table 1; Fig. 1D; marginal R^2 = 0.029).

3.2. Identifying which factors affect haemolysis in the plasma of roe deer populations

In our roe deer plasma samples (n = 707), the levels of haemoglobin varied between 7.5 and 469.3 mg/dl, with a mean value of 38.4 ± 37.2 mg/dl, and a median value of 28.6 mg/dl.

Following model selection, the model that best explained the variability in log-transformed haemoglobin concentrations among roe deer plasma samples included only age, but not sex, body mass or population (Table S1). Haemoglobin levels were negatively associated with age (Table 2). Hence, younger individuals tended to exhibit higher haemoglobin levels (Fig. 2).

3.3. Effect of haemolysis in plasma samples on the evaluation of redox status

To investigate whether the degree of haemolysis in plasma affects the assessement of oxidative stress biomarkers patterns in the two wild populations of roe deer, we used MDA (as oxidative damage) and SOD (as antioxidant), two markers for which the *in vitro* approach showed that haemolysis influenced significantly their measurements (see Fig. 1).

When excluding haemoglobin from the models, the retained model for log-transformed MDA levels included the effect of population, with roe deer from Trois-Fontaines displaying higher oxidative damages on lipids than those from Chizé (Table 3a). Concerning plasma SOD activity, the retained model included the effects of population, but also individual age, sex, and body mass. Roe deer from Chizé exhibited higher SOD activity than those from Trois-Fontaines. Additionally, SOD activity was higher in males, in younger, and in lighter individuals (Table 3a).

Adding haemoglobin concentration as a covariate in the models revealed a positive association between this covariate and both MDA and SOD (Table 3b), supporting findings of our *in vitro* approach (Fig. 1). AIC of the models including haemoglobin were clearly lower than those of the corresponding models without haemoglobin (difference > 48, Table S2). The marginal R^2, expressing the effect of fixed factors, increased moderately (from 0.032 to 0.063) for MDA, but stepped up from 0.054 to 0.223 for SOD. For SOD, haemoglobin thus explained more variability than other fixed effects. However, adding haemoglobin had no major effect on the model structure for both biomarkers, *i.e.* the same fixed effects were selected by the model selection procedure whether or not haemoglobin was included in the initial model (Table 3b). Adding haemoglobin concentration as covariate also had no striking impact on the estimates of the parameters of redox status (Table 3).

4. Discussion

The primary objective of this study was to investigate the potential methodological bias induced by haemolysis in plasma, assessing its impact on our understanding of the relationships between oxidative stress markers and both individual and environmental factors. Using roe deer samples from two wild populations, we found that, in our study context, haemolysis i) leads to a net overestimation of oxidative damages and antioxidant defences in five out of six biomarkers; ii) is more pronounced in younger than in older individuals; and iii) when taken into account in the analyses, increases the goodness of fit of explanatory models without significantly altering the conclusions about the relationships between oxidative stress markers and individual factors.

4.1. Effect of haemoglobin on oxidative stress and antioxidant markers

Out of the six tested biomarkers that are commonly used in eco-evolutionary studies for characterising redox status, all but one (*i.e.* dROMs) showed a positive and linear association with haemoglobin concentration in RBC-enriched plasma samples. In other words, the haemolysis in plasma resulted in an overestimation of both oxidative damage and antioxidant defence values. The mechanism by which haemolysis might artificially inflate oxidative stress markers may have different origins. Firstly, the coloration caused by haemoglobin or its derivatives can directly influence the measurements, in particular when spectrophotometric readings are performed at wavelength close to the haemoglobin absorption peaks (typically 415, 540 and 570 nm) (Lippi et al., 2008). This effect may be less pronounced using alternative analytical methods such as high-performance liquid chromatography (HPLC), enzymatic- or radio-immunoassays (EIA or RIA), or

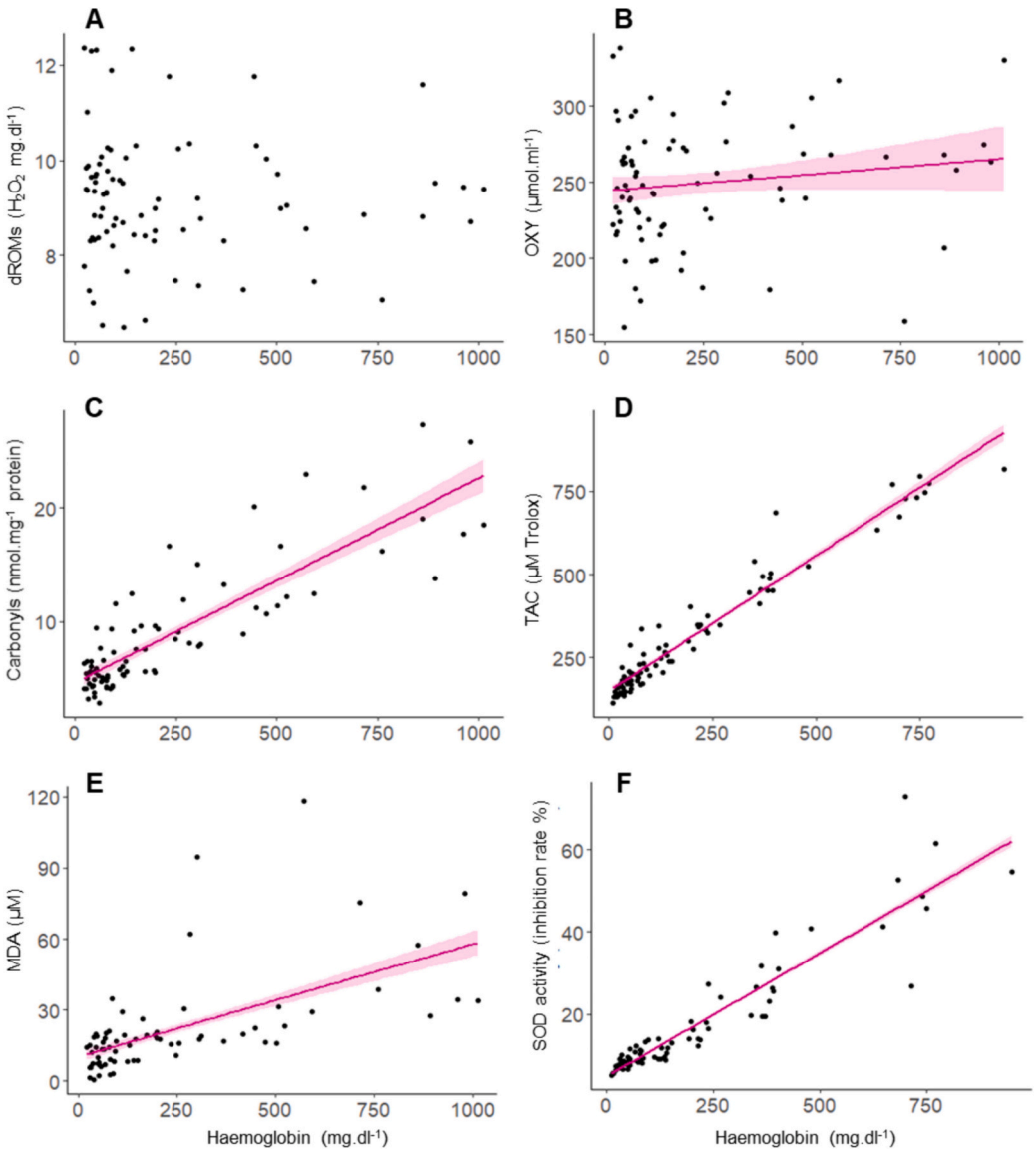

Fig. 1. Effects of haemoglobin concentration, resulting from *in vitro* adding of lysed RBCs, on the concentrations of oxidative damage (left panels) and antioxidant (right panels) biomarkers in roe deer plasma. The graphs show measurements from samples collected on 10 individuals. For each of them, 8 plasma sub-samples with increasing degrees of haemolysis were obtained by experimental enrichment with haemolysed RBCs. When statistically significant, the linear regression is shown with a 95 % confidence interval (pink shade). Effect of haemoglobin levels on (A) dROM levels ($n = 80$), (B) OXY levels ($n = 80$), (C) Protein carbonyls levels ($n = 80$), (D) Total antioxidant capacity ($n = 80$), (E) Malondialdehyde levels ($n = 71$), and (F) Superoxide dismutase activity ($n = 76$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Parameter estimates of best linear mixed effects model describing the log-transformed haemoglobin concentrations as a function of age in roe deer. Parameter estimates are given with 95 % confidence intervals (95 %CI). Models included individual identity and assay plate number as random effects.

Variables	Estimate	95 %CI
Intercept	3.610	[3.528; 3.691]
Age (year)	−0.046	[−0.060; −0.033]
Marginal R^2	0.060	
Condition R^2	0.142	

fluorometric assays. Secondly, several intracellular molecules, other than haemoglobin released by RBC rupture, may interfere with reagents and/or reaction products of the assay, as observed in other biochemical and clinical assays (*e.g.* in ewes, Morris et al., 2002; in sows, Theil et al., 2013; in wild birds, Roman et al., 2009; in fish, Mirghaed et al., 2017). Thirdly, haemoglobin is a major source of ROS due to its ability to auto-oxidize and generate superoxide anion, hydroxyl anion, and hydrogen peroxide. When haemoglobin auto-oxidation occurs at the extracellular level following haemolysis, the release of superoxide anions causes major oxidative damages whose physio-pathological consequences are well recognized *in vivo* (Rifkind et al., 2015), but whose effects on oxidative damage markers *in vitro* are, regrettably, ignored. Finally, some markers of the redox homeostasis are much more concentrated in RBCs than in plasma, so that even a small fraction of lysed cells can significantly alter the plasma composition (Lippi et al., 2008). For example, in humans, the concentration of reduced glutathione (GSH), the main thiol-based antioxidant, is about 1000 times higher in the cytoplasm of RBCs than in plasma (Giustarini et al., 2017). Ironically, the measurement of plasma GSH has been proposed as a tool to quantify haemolysis and is used as a marker of plasma quality (Tomin et al., 2021). It should be noted that third and fourth points discussed above do not depend on the analytical method used, and although our data are all

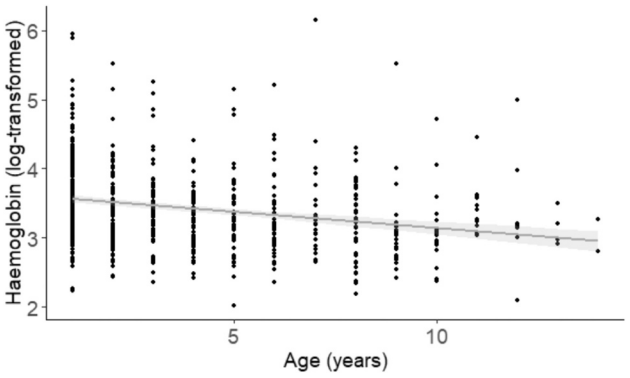

Fig. 2. Effects of age on log-transformed haemoglobin levels in roe deer (n = 707). Points represent raw data, and the line the predictions from the selected model with 95 % confidence interval.

Table 3
Parameter estimates of selected linear mixed effects models describing the redox status as a function of (a) age, population, sex and body mass, or (b) haemoglobin, age, population, sex and body mass in roe deer. For sex and population variables, intercept included females and individuals from Chizé, respectively. Parameter estimates are given with 95 % confidence intervals (95 %CI). Models included individual identity and assay plate number as random effects.

Variables	log-MDA (n = 702)		SOD (n = 702)	
	Estimate	95 %CI	Estimate	95 %CI
(a)				
Intercept	2.198	[2.053; 2.343]	72.376	[69.586; 75.166]
Age (year)			−0.488	[−0.649; −0.328]
Population (TF)	0.175	[0.123; 0.227]	−1.895	[−2.932; −0.859]
Sex (males)			1.293	[0.303; 2.282]
Body mass (kg)			−0.420	[−0.741; −0.100]
Marginal R^2	0.032		0.054	
Conditional R^2	0.638		0.515	
(b)				
Intercept	2.110	[1.964; 2.256]	68.090	[65.427; 70.753]
Haemoglobin	0.002	[0.002; 0.003]	0.096	[0.085; 0.107]
Age (year)			−0.344	[−0.482; −0.206]
Population (TF)	0.176	[0.125; 0.227]	−1.912	[−2.798; −1.025]
Sex (males)			1.495	[0.647; 2.343]
Body mass (kg)			−0.347	[−0.610; −0.084]
Marginal R^2	0.063		0.223	
Conditional R^2	0.676		0.672	

from spectrophotometric assays, effects of haemolysis on oxidative stress markers are also expected with other analytical methods (HPLC, RIA, fluorimetric assays).

The relative contribution of the different mechanisms by which haemolysis can interfere with markers of the oxidative balance is difficult to apprehend and may depend on the specificity of each marker and assay. For example, MDA concentration in RBCs are known to be of the same order of magnitude to those in plasma (Richard et al., 1992), so the linear increase of MDA with haemolysis that we evidenced here is unlikely due to changes in the resulting plasma MDA concentration, but rather to interferences with spectrophotometric readings. Indeed, the wavelength used to measure MDA spectrophotometrically lies within the haemoglobin absorption peak (570 nm). This is one of the reasons why it has been suggested to avoid determining this marker spectrophotometrically (Halliwell and Gutteridge, 2015; Murphy et al., 2022). Note however, that a similar correlation between artificial haemolysis and MDA has been previously observed in human plasma using other analytical method (GC–MS/MS) (Dreiβigacker et al., 2010). Similarly, several DNPH-based protocols for carbonyl assay have also been shown to be sensitive to the presence of chromogens such as haemoglobin (Fagan et al., 1999). In contrast, the colorimetric assay of superoxide dismutase activity includes a blank for each sample to account for red coloration in plasma (see SOD booklet *Sigma-Aldrich, reference number: CS0009*), so the sharp increase in SOD activity with haemolysis suggests that plasma is enriched with cytosolic SOD1 and/or mitochondrial SOD2 as blood cells break up and release their contents. Finally, we can expect the interferences between haemolysis and markers to be complex and unpredictable, as it seems to be the case for dROMs. Indeed, levels in plasma enriched with lysed RBCs were highly dispersed and we detected no clear tendency for an increase or a decrease with increasing haemolysis degree. The debate on the relevance, specificity and limitations of oxidative stress markers is not the focus of this article and has been addressed elsewhere (Hõrak and Cohen, 2010; Tsikas, 2017). However, our results show that the sensitivity of oxidative stress markers to haemolysis is highly variable from one marker to another, and maybe even from one species to another, suggesting that it must be considered on a case-by-case basis, whatever the analytical method used.

While studies documenting a covariation between haemolysis and plasma biomarkers of oxidative stress are rare and limited to the medical literature, our results are in accordance with them. For instance, a significant positive correlation between *in vivo* haemolysis and plasma MDA concentrations was found in a clinical study on humans who had undergone intensive physical exercise (Dreiβigacker et al., 2010). Similarly, higher plasma haemoglobin concentrations were associated with increased plasma protein carbonyl levels in human patients with sickle cell disease (Kupesiz et al., 2012). These studies also suggest that a number of individual variables influence haemolysis *in vivo*, posing a challenge for studies on animals in natural conditions where the physiopathophysiological status of the studied individuals is generally unknown. Nevertheless, *in vitro* haemolysis is expected to occur more frequently in field studies than in laboratory conditions, where the conditions under which blood samples are collected, handled or transported can be precarious, and may favour the breakdown of the most sensitive blood cells (see below). Still, and although it remains unclear whether this haemolysis is mainly of *in vivo* or *in vitro* origin, our results show that it affects certain categories of individuals. Below, we discuss the observed patterns of haemolysis in wild roe deer and the possible environmental (low quality *versus* high quality food resources) and individual (age, body mass and sex) variables shaping these patterns.

4.2. Factors affecting patterns of haemolysis in the plasma of wild roe deer populations

We assessed patterns of haemolysis in plasma samples collected in two roe deer populations by measuring their haemoglobin content. To our knowledge, comprehensive information on the patterns of haemolysis in wild animals is lacking, and current data in the literature is derived from a few studies on domestic or captive animals, and mostly from human biomedical studies. For instance, the mean haemoglobin concentration was 28 ± 10 mg/dl in the serum of healthy pigs (Dorner et al., 1983), while it was 7 ± 3 mg/dl in the plasma of captive bar-headed geese, and 8 ± 5 mg/dl in that of captive black kites (Roman et al., 2009). In humans, samples containing less than 5 mg/dl free haemoglobin are typically considered as non-haemolysed (Lippi et al., 2011b).

Among the factors that may influence haemolysis in plasma samples, we tested whether the environmental context (low quality *versus* high quality food resources) could influence the RBC membranes susceptibility to haemolysis, and ultimately the occurrence of haemolysis in plasma samples, by comparing roe deer from two populations residing in habitats of contrasted quality (Chizé *vs.* Trois-Fontaines study sites). Our results do not provide support for the hypothesized influence of the resources quality on RBC membrane susceptibility to haemolysis. However, we focused solely on the study site effect, whereas more specific variables, such as climate or vegetation, could be used to explore more precisely environmental-related differences in haemolysis patterns. When exploring the influence of individual variables, we found

that neither body mass nor sex emerged as significant explanatory factors in models predicting haemolysis patterns. This suggests that there is neither difference in haemolysis susceptibility between lighter and heavier individuals, nor between males and females, a result that stands in contrast with observations in humans, where the prevalence of haemolysis is typically lower in women compared to men (Kanias et al., 2017; Liu et al., 2016). Conversely, our data reveals a negative association between haemoglobin levels and age, indicating that younger individuals exhibit higher haemolysis in their plasma. This pattern aligns with observations in humans, where haemolysis prevalence was also reported to be lower in elderly individuals, but more pronounced in blood samples collected from paediatrics departments (Liu et al., 2016). This result also echoes previous findings showing that membrane resistance of RBC to trauma increases with age in humans (Penha-Silva et al., 2007).

Overall, our results show that susceptibility to haemolysis may depend on individual characteristics, making it crucial to identify factors promoting haemolysis to minimize it. Haemolysis can occur both *in vivo* and *in vitro*, at different experimental stages. Firstly, the stress associated to capture, restraint and handling has been reported to contribute to fluctuations in many haematological parameters, including RBC count and haemoglobin concentrations. It is known from human biomedical research that exercise-induced stress can cause haemolysis and substantially influence carbonyl content (Şentürk et al., 2005). Hence, refining capture protocols to minimize stress, struggle and/or effort, such as use of chemical immobilisation (Cross et al., 1988; Marco and Lavín, 1999; Montané et al., 2003), may be one solution to reduce the occurrence or severity of *in vivo* haemolysis. Secondly, the integrity of RBCs during blood collection can be compromised by various factors related to sampling techniques and conditions (Lippi et al., 2011b). Beyond the presumed proficiency of the blood sampling operator, both the sampling technique and the equipment play a crucial role in the occurrence of haemolysis. For instance, human blood samples obtained through vein puncture typically exhibit less haemolysis than those collected with a catheter, especially when using butterfly needles (Heireman et al., 2017). The choice of needle and collection tube also matters, with thinner needles and larger tubes causing more trauma to RBCs (Carraro et al., 2000; Heireman et al., 2017; Lippi et al., 2011a). Minimizing the time between sampling and centrifugation is also critical, as haemolysis can happen after blood sampling is completed (Heireman et al., 2017). Lastly, conservation method and time before assessment can alter samples quality (Coker, 2002; Lippi et al., 2011b). However, rapidly processing the samples and freezing them at −80 °C upon collection, as done in the present study, may effectively limit post-collection haemolysis. It should be noted that other factors may also influence haemoglobin patterns in plasma samples, such as whole blood haemoglobin concentrations (Jenks et al., 2019), which we did not control for here and this may be a limitation of this study.

4.3. Effect of haemolysis in plasma samples on the evaluation of redox status

Our findings indicate that haemoglobin measured in plasma contributes to explain the observed inter-individual variability for two different markers (*i.e.* MDA for oxidative damages and SOD for antioxidant defences), as taking haemoglobin into account as a covariate clearly improves the goodness of fit of explanatory models. Given that haemolysis leads to an overestimation of oxidative stress biomarkers values, and that the occurrence of haemolysis is age-related in our roe deer populations, it could potentially reveal an age effect on the response variable, when it is actually mirroring haemolysis influence. This phenomenon should become more likely with increased haemolysis in plasma samples. In our study, blood samples were collected using large-diameter needles (width of 1.1 mm) to minimize trauma to RBCs, and were processed immediately after collection on site. They were neither transported nor stored prior to centrifugation, in order to minimize mechanical stress on RBC and limit *in vitro* haemolysis. As a consequence, with a mean of plasma haemoglobin concentrations of 38.4 ± 37.2 mg/dl, our dataset includes only a few haemolysed samples (0.7 % of the total samples fall under the category termed "grossly haemolysed", *i.e.* ≥ 200 mg/dl, according to Lippi et al., 2011a, 2011b) in a relatively large sample size. Their inclusion in statistical models poses therefore minimal risk. Nevertheless, our data clearly suggests that higher levels of haemolysis would increase the likelihood of this masking effect and should not be overlooked. Indeed, correlation coefficients between haemoglobin concentrations and the different oxidative stress biomarkers revealed strong correlations in the *in vivo* experiment, as well as in the *in vitro* experiment where haemoglobin concentrations have been truncated to the maximum value obtained in our natural populations. This was true for all biomarkers that had strong correlations in the original *in vitro* experiment, except OXY (see Table S3).

The results of the present study highlight an overestimation of oxidative stress marker values due to haemolysis in plasma samples. Individual traits and environmental conditions did not reveal any effect on haemolysis, except age, as younger individuals exhibited higher haemolysis in the plasma than older ones. Finally, when haemolysis was considered in the analyses, it increased the goodness of fit of explanatory models without significantly altering the conclusions about the relationships between oxidative stress markers and individual factors. The risk of haemolysis having an impact on our comprehension of the factors governing patterns of oxidative stress emphasizes the importance of measuring, and subsequently integrating, haemoglobin levels into statistical analyses. In retrospect, these observations somehow question whether the relationship between plasma markers of oxidative stress with age classically observed in natural populations (Metcalfe and Alonso-Alvarez, 2010) is not influenced to some extent to an effect of age on the degree of haemolysis in plasma. In particular, our data suggests that the bias associated with haemolysis is more pronounced in early life stages, suggesting that previously observed decreases in plasma oxidative stress markers with age may need to be reassessed. Furthermore, individual and environmental characteristics are used most of the time in more complex analyses, investigating for example the influence of oxidative stress on life history traits. Consequently, while our findings may pertain specifically to the markers assessed in the present study, it appears that haemolysis has the potential to significantly alter results interpretation. This is all more the more important as it affects all areas of research and fieldwork involving the assessment of plasmatic markers. We advise to systematically questioning the effects of haemolysis on plasma oxidative stress markers in future studies. In particular, we recommend identifying which individual or environmental factors may explain patterns of haemolysis and, where appropriate, controlling for the degree of haemolysis in analyses when studying biomarkers of oxidative stress. This is a novel approach, as it is often advised to remove the haemolysed samples from the dataset (for *e.g.* Perez-Montero et al., 2023), which may certainly be the best option when the sampling can be easily repeated. It may also be possible to use haemoglobin as a correction factor for overall results upstream of the models. Finally, these recommendations are extendable to other physiological markers measured in plasma, as they serve both as a quality control measure to ensure data integrity, and as a means of improving the accuracy and interpretability of statistical models. Further studies should incorporate the use of additional biomarkers and involve different species to extend the results of this study. We therefore recommend that the impact of haemolysis on biomarkers and species be evaluated to ensure more accurate and generalizable findings.

Funding

This work was supported by a grant from the Agence Nationale de la Recherche (ANR-15-CE32-0002-01 to J.F.L.). The Trois-Fontaines population is part of the long-term Studies in Ecology and Evolution (SEE-

Life) program of the CNRS.

CRediT authorship contribution statement

Amandine Herrada: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Pauline Vuarin:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **François Débias:** Writing – review & editing, Data curation. **Alexia Gache:** Writing – review & editing, Methodology, Data curation. **Philippe Veber:** Writing – review & editing, Formal analysis. **Maryline Pellerin:** Writing – review & editing, Project administration. **Louise Cheynel:** Writing – review & editing, Methodology, Data curation. **Jean-François Lemaître:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Emmanuelle Gilot-Fromont:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Benjamin Rey:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is available upon request from the corresponding author.

Acknowledgments

We would like to thank Gilles Capron, Stéphane Chabot and Claude Warnant of the OFB, and all the field volunteers without whom the capture of roe deer would not have been possible.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111750.

References

Aloise King, E.D., Garratt, M., Brooks, R., 2013. Manipulating reproductive effort leads to changes in female reproductive scheduling but not oxidative stress. Ecol. Evol. 3, 4161–4171. https://doi.org/10.1002/ece3.786.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Sorci, G., 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction: oxidative stress as a cost of reproduction. Ecol. Lett. 7, 363–368. https://doi.org/10.1111/j.1461-0248.2004.00594.x.

Alonso-Alvarez, C., Canelo, T., Romero-Haro, A.Á., 2017. The oxidative cost of reproduction: theoretical questions and alternative mechanisms. BioScience 67, 258–270. https://doi.org/10.1093/biosci/biw176.

Arnold, T.W., 2010. Uninformative parameters and model selection using Akaike's information criterion. J. Wildl. Manag. 74, 1175–1178. https://doi.org/10.1111/j.1937-2817.2010.tb01236.x.

Bartoń, K., 2013. MuMIn: Multi-model inference, R package version 1.10.0.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67. https://doi.org/10.18637/jss.v067.i01.

Blount, J.D., Vitikainen, E.I.K., Stott, I., Cant, M.A., 2016. Oxidative shielding and the cost of reproduction: oxidative shielding and the cost of reproduction. Biol. Rev. 91, 483–497. https://doi.org/10.1111/brv.12179.

Burnham, K.P., Anderson, D.R., 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach, 2nd ed. Springer, New York.

Burton, G.J., Jauniaux, E., 2011. Oxidative stress. Best Pracr Res Clin Obstet Gynaecol, Placental Bed & Maternal - Fetal Disorders 25, 287–299. https://doi.org/10.1016/j.bpobgyn.2010.10.016.

Carraro, P., Servidio, G., Plebani, M., 2000. Hemolyzed specimens: a reason for rejection or a clinical challenge? Clin. Chem. 46, 306–307. https://doi.org/10.1093/clinchem/46.2.306.

Christensen, L.L., Selman, C., Blount, J.D., Pilkington, J.G., Watt, K.A., Pemberton, J.M., Reid, J.M., Nussey, D.H., 2015. Plasma markers of oxidative stress are uncorrelated in a wild mammal. Ecol. Evol. 5, 5096–5108. https://doi.org/10.1002/ece3.1771.

Cohen, A., Klasing, K., Ricklefs, R., 2007. Measuring circulating antioxidants in wild birds. Comp. Biochem. Physiol. Part B 147, 110–121. https://doi.org/10.1016/j.cbpb.2006.12.015.

Coker, S., 2002. Red blood cell hemolysis during processing. Transfus. Med. Rev. 16, 46–60. https://doi.org/10.1053/tmrv.2002.29404.

Costantini, D., 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett. 11, 1238–1251. https://doi.org/10.1111/j.1461-0248.2008.01246.x.

Costantini, D., 2011. On the measurement of circulating antioxidant capacity and the nightmare of uric acid. Methods Ecol. Evol. 2, 321–325. https://doi.org/10.1111/j.2041-210X.2010.00080.x.

Costantini, D., 2022. A meta-analysis of impacts of immune response and infection on oxidative status in vertebrates. Conserv. Physiol. 10, coac018. https://doi.org/10.1093/conphys/coac018.

Costantini, D., Casasole, G., Eens, M., 2014. Does reproduction protect against oxidative stress? J. Exp. Biol. https://doi.org/10.1242/jeb.114116 jeb.114116.

Cross, J.P., Mackintosh, C.G., Griffin, J.F.T., 1988. Effect of physical restraint and xylazine sedation on haematological values in red deer (*Cervus elaphus*). Res. Vet. Sci. 45, 281–286. https://doi.org/10.1016/S0034-5288(18)30951-2.

Czerska, M., Mikołajewska, K., Zieliński, M., Gromadzińska, J., Wąsowicz, W., 2015. Today's oxidative stress markers. Med. Pr. 66. https://doi.org/10.13075/mp.5893.00137.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2.

Delianu, C., Hurjui, L.L., Moscalu, M., Tărniceriu, C.C., Hurjui, I., Bădulescu, O.-V., Foia, L., 2020. Hemolysis - at the border of the controversy or malpractice. Med. Surg. J. 124, 298–306.

Dorner, J.L., Hoffmann, W.E., Filipov, M.M., 1983. Effect of *in vitro* hemolysis on values for certain porcine serum constituents. Vet. Clin. Pathol. 12, 15–19. https://doi.org/10.1111/j.1939-165X.1983.tb00829.x.

Douhard, M., Gaillard, J.-M., Delorme, D., Capron, G., Duncan, P., Klein, F., Bonenfant, C., 2013. Variation in adult body mass of roe deer: early environmental conditions influence early and late body growth of females. Ecology 94, 1805–1814. https://doi.org/10.1890/13-0034.1.

Douhard, F., Gaillard, J.-M., Pellerin, M., Jacob, L., Lemaître, J.-F., 2017. The cost of growing large: costs of post-weaning growth on body mass senescence in a wild mammal. Oikos 126, 1329–1338. https://doi.org/10.1111/oik.04421.

Dowling, D.K., Simmons, L.W., 2009. Reactive oxygen species as universal constraints in life-history evolution. Proc. R. Soc. B 276, 1737–1745. https://doi.org/10.1098/rspb.2008.1791.

Dreißigacker, U., Suchy, M.-T., Maassen, N., Tsikas, D., 2010. Human plasma concentrations of malondialdehyde (MDA) and the F2-isoprostane 15(S)-8-iso-PGF2α may be markedly compromised by hemolysis: evidence by GC-MS/MS and potential analytical and biological ramifications. Clin. Biochem. 43, 159–167. https://doi.org/10.1016/j.clinbiochem.2009.10.002.

Fagan, J.M., Sleczka, B.G., Sohar, I., 1999. Quantitation of oxidative damage to tissue proteins. Int. J. Biochem. Cell Biol. 31, 751–757. https://doi.org/10.1016/S1357-2725(99)00034-5.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247. https://doi.org/10.1038/35041687.

Flerov, K.K., 1952. Fauna of USSR, Mammals Vol. 1, No. 2. Musk Deer and Deer. USSR Academia Science.

Fraser, S.T., Midwinter, R.G., Berger, B.S., Stocker, R., 2011. Heme oxygenase-1: a critical link between iron metabolism, erythropoiesis, and development. Adv. Hematol. 2011, e473709. https://doi.org/10.1155/2011/473709.

Gaillard, J.-M., Delorme, D., Jean-Marie, B., Van Laere, G., Boisaubert, B., Pradel, R., 1993. Roe deer survival patterns: a comparative analysis of contrasting populations. J. Anim. Ecol. 62, 778–791. https://doi.org/10.2307/5396.

Garratt, M., McArdle, F., Stockley, P., Vasilaki, A., Beynon, R.J., Jackson, M.J., Hurst, J. L., 2012. Tissue-dependent changes in oxidative damage with male reproductive effort in house mice. Funct. Ecol. 26, 423–433. https://doi.org/10.1111/j.1365-2435.2011.01952.x.

Gilot-Fromont, E., Jégo, M., Bonenfant, C., Gibert, P., Rannou, B., Klein, F., Gaillard, J.-M., 2012. Immune phenotype and body condition in roe deer: individuals with high body condition have different, not stronger immunity. PLoS One 7, e45576. https://doi.org/10.1371/journal.pone.0045576.

Giustarini, D., Colombo, G., Garavaglia, M.L., Astori, E., Portinaro, N.M., Reggiani, F., Badalamenti, S., Aloisi, A.M., Santucci, A., Rossi, R., Milzani, A., Dalle-Donne, I., 2017. Assessment of glutathione/glutathione disulphide ratio and S-glutathionylated proteins in human blood, solid tissues, and cultured cells. Free Radic. Biol. Med. 112, 360–375. https://doi.org/10.1016/j.freeradbiomed.2017.08.008.

Halliwell, B., Gutteridge, J.M.C., 2015. Free Radicals in Biology and Medicine. Oxford University Press.

Heireman, L., Van Geel, P., Musger, L., Heylen, E., Uyttenbroeck, W., Mahieu, B., 2017. Causes, consequences and management of sample hemolysis in the clinical laboratory. Clin. Biochem. 50, 1317–1322. https://doi.org/10.1016/j.clinbiochem.2017.09.013.

Heyer, N.J., Derzon, J.H., Winges, L., Shaw, C., Mass, D., Snyder, S.R., Epner, P., Nichols, J.H., Gayken, J.A., Ernst, D., Liebow, E.B., 2012. Effectiveness of practices to reduce blood sample hemolysis in EDs: a laboratory medicine best practices systematic review and meta-analysis. Clin. Biochem. 45, 1012–1032. https://doi.org/10.1016/j.clinbiochem.2012.08.002.

Hõrak, P., Cohen, A., 2010. How to measure oxidative stress in an ecological context: methodological and statistical issues. Funct. Ecol. 24, 960–970. https://doi.org/10.1111/j.1365-2435.2010.01755.x.

Jenks, C.L., Zia, A., Venkataraman, R., Raman, L., 2019. High hemoglobin is an independent risk factor for the development of hemolysis during pediatric extracorporeal life support. J. Intensive Care Med. 34, 259–264. https://doi.org/10.1177/0885066617708992.

Kanias, T., Lanteri, M.C., Page, G.P., Guo, Y., Endres, S.M., Stone, M., Keating, S., Mast, A.E., Cable, R.G., Triulzi, D.J., Kiss, J.E., Murphy, E.L., Kleinman, S., Busch, M. P., Gladwin, M.T., for the National Heart, L., and Blood Institute Recipient Epidemiology Donor Evaluation Study III (REDS-III) Program, 2017. Ethnicity, sex, and age are determinants of red blood cell storage and stress hemolysis: results of the REDS-III RBC-omics study. Blood Adv. 1, 1132–1141. https://doi.org/10.1182/bloodadvances.2017004820.

Kupesiz, A., Celmeli, G., Dogan, S., Antmen, B., Aslan, M., 2012. The effect of hemolysis on plasma oxidation and nitration in patients with sickle cell disease. Free Radic. Res. 46, 883–890. https://doi.org/10.3109/10715762.2012.686037.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.-G., Ahn, B.-W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. In: Meth. Enzymol. Elsevier, pp. 464–478. https://doi.org/10.1016/0076-6879(90)86141-H.

Lippi, G., Blanckaert, N., Bonini, P., Green, S., Kitchen, S., Palicka, V., Vassault, A.J., Plebani, M., 2008. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin. Chem. Lab. Med. 46. https://doi.org/10.1515/CCLM.2008.170.

Lippi, G., Avanzini, P., Da Pavesi, F., Bardi, M., Ippolito, L., Aloe, R., Favaloro, E.J., 2011a. Studies on in vitro hemolysis and utility of corrective formulas for reporting results on hemolyzed specimens. Biochem. Med. 297–305. https://doi.org/10.11613/BM.2011.040.

Lippi, G., Plebani, M., Di Somma, S., Cervellin, G., 2011b. Hemolyzed specimens: a major challenge for emergency departments and clinical laboratories. Crit. Rev. Clin. Lab. Sci. 48, 143–153. https://doi.org/10.3109/10408363.2011.600228.

Liu, D., Li, Y.-X., Huang, Y., 2016. The prevalence of hemolysis - a survey using hemolysis index. Clin. Chem. Lab. Med. 55. https://doi.org/10.1515/cclm-2016-0698.

Marco, I., Lavín, S., 1999. Effect of the method of capture on the haematology and blood chemistry of red deer (*Cervus elaphus*). Res. Vet. Sci. 66, 81–84. https://doi.org/10.1053/rvsc.1998.0248.

McGraw, K.J., Cohen, A.A., Costantini, D., Hõrak, P., 2010. The ecological significance of antioxidants and oxidative stress: a marriage between mechanistic and functional perspectives: editorial. Funct. Ecol. 24, 947–949. https://doi.org/10.1111/j.1365-2435.2010.01772.x.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24, 984–996. https://doi.org/10.1111/j.1365-2435.2010.01750.x.

Metcalfe, N.B., Monaghan, P., 2003. Growth versus lifespan: perspectives from evolutionary ecology. Exp. Gerontol. 38, 935–940. https://doi.org/10.1016/S0531-5565(03)00159-1.

Metcalfe, N.B., Monaghan, P., 2013. Does reproduction cause oxidative stress? An open question. Trends Ecol. Evol. 28, 347–350. https://doi.org/10.1016/j.tree.2013.01.015.

Milner, J.M., Stien, A., Irvine, R.J., Albon, S.D., Langvatn, R., Ropstad, E., 2003. Body condition in Svalbard reindeer and the use of blood parameters as indicators of condition and fitness. Can. J. Zool. 81, 1566–1578. https://doi.org/10.1139/z03-152.

Mirghaed, A.T., Ghelichpour, M., Hoseini, S.M., Amini, K., 2017. Hemolysis interference in measuring fish plasma biochemical indicators. Fish Physiol. Biochem. 43, 1143–1151. https://doi.org/10.1007/s10695-017-0359-y.

Monaghan, P., Metcalfe, N.B., Torres, R., 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol. Lett. 12, 75–92. https://doi.org/10.1111/j.1461-0248.2008.01258.x.

Montané, J., Marco, I., López-Olvera, J., Perpiñán, D., Manteca, X., Lavín, S., 2003. Effects of Acepromazine on capture stress in roe deer (*Capreolus capreolus*). J. Wildl. Dis. 39, 375–386. https://doi.org/10.7589/0090-3558-39.2.375.

Morris, J.D., Fernandez, J.M., Chapa, A.M., Gentry, L.R., Thorn, K.E., Weick, T.M., 2002. Effects of sample handling, processing, storage, and hemolysis on measurements of key energy metabolites in ovine blood. Small Rumin. Res. 43, 157–166. https://doi.org/10.1016/S0921-4488(01)00266-8.

Murphy, M.P., Bayir, H., Belousov, V., Chang, C.J., Davies, K.J.A., Davies, M.J., Dick, T. P., Finkel, T., Forman, H.J., Janssen-Heininger, Y., Gems, D., Kagan, V.E., Kalyanaraman, B., Larsson, N.-G., Milne, G.L., Nyström, T., Poulsen, H.E., Radi, R., Van Remmen, H., Schumacker, P.T., Thornalley, P.J., Toyokuni, S., Winterbourn, C. C., Yin, H., Halliwell, B., 2022. Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. Nat. Metab. 4, 651–662. https://doi.org/10.1038/s42255-022-00591-z.

Norte, A.C., Sheldon, B., Sousa, J.P., Ramos, J.A., 2008. Repeatability and method-dependent variation of blood parameters in wild-caught great tits *Parus major*. Acta Ornithol. 43, 65–75. https://doi.org/10.3161/000164508X345347.

Nussey, D.H., Pemberton, J.M., Pilkington, J.G., Blount, J.D., 2009. Life history correlates of oxidative damage in a free-living mammal population. Funct. Ecol. 23, 809–817. https://doi.org/10.1111/j.1365-2435.2009.01555.x.

Ołdakowski, Ł., Taylor, J.R.E., 2018. Oxidative damage and antioxidant defense are assay and tissue-dependent both in captive and wild-caught bank voles (*Myodes glareolus*) before and after reproduction. Ecol. Evol. 8, 7543–7552. https://doi.org/10.1002/ece3.4187.

Ołdakowski, Ł., Wasiluk, A., Sadowska, E.T., Koteja, P., Taylor, J.R.E., 2015. Reproduction is not costly in terms of oxidative stress. J. Exp. Biol. https://doi.org/10.1242/jeb.126557 jeb.126557.

Pawlik-Sobecka, L., Sołkiewicz, K., Kokot, I., Kiraga, A., Płaczkowska, S., Schlichtinger, A.M., Kratz, E.M., 2020. The influence of serum sample storage conditions on selected laboratory parameters related to oxidative stress: a preliminary study. Diagnostics 10, 51. https://doi.org/10.3390/diagnostics10010051.

Penha-Silva, N., Firmino, C.B., De Freitas Reis, F.G., Huss, J.C.D.C., De Souza, T.M.T., De Freitas, M.V., Netto, R.D.C.M., 2007. Influence of age on the stability of human erythrocyte membranes. Mech. Ageing Dev. 128, 444–449. https://doi.org/10.1016/j.mad.2007.06.007.

Perez-Montero, B., Fermin-Rodriguez, M.L., Miro, G., de Juan, L., Cruz-Lopez, F., 2023. Hemolysis, icterus and lipemia interfere with the determination of two oxidative stress biomarkers in canine serum. BMC Vet. Res. 19, 172. https://doi.org/10.1186/s12917-023-03740-y.

Pettorelli, N., Gaillard, J.-M., Mysterud, A., Duncan, P., Chr Stenseth, N., Delorme, D., Van Laere, G., Toïgo, C., Klein, F., 2006. Using a proxy of plant productivity (NDVI) to find key periods for animal performance: the case of roe deer. Oikos 112, 565–572. https://doi.org/10.1111/j.0030-1299.2006.14447.x.

R core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria [WWW Document]. URL. https://www.r-project.org/ (accessed 5.26.22).

Rey, B., Dégletagne, C., Bodennec, J., Monternier, P.-A., Mortz, M., Roussel, D., Romestaing, C., Rouanet, J.-L., Tornos, J., Duchamp, C., 2016. Hormetic response triggers multifaceted anti-oxidant strategies in immature king penguins (*Aptenodytes patagonicus*). Free Radic. Biol. Med. 97, 577–587. https://doi.org/10.1016/j.freeradbiomed.2016.07.015.

Richard, M.J., Guiraud, P., Meo, J., Favier, A., 1992. High-performance liquid chromatographic separation of malondialdehyde—thiobarbituric acid adduct in biological materials (plasma and human cells) using a commercially available reagent. J. Chromatogr. B 577, 9–18. https://doi.org/10.1016/0378-4347(92)80593-F.

Rifkind, J.M., Mohanty, J.G., Nagababu, E., 2015. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. Front. Physiol. 5.

Roman, Y., Bomsel-Demontoy, M.-C., Levrier, J., Chaste-Duvernoy, D., Jalme, M.S., 2009. Effect of hemolysis on plasma protein levels and plasma electrophoresis in birds. J. Wildl. Dis. 45, 73–80. https://doi.org/10.7589/0090-3558-45.1.73.

Selman, C., Blount, J., Nussey, D., Speakman, J., 2012. Oxidative damage, ageing, and life-history evolution: where now? Trends Ecol. Evol. 27, 570–577. https://doi.org/10.1016/j.tree.2012.06.006.

Şentürk, Ü.K., Gündüz, F., Kuru, O., Koçer, G., Özkaya, Y.G., Yeşilkaya, A., Bor-Küçükatay, M., Üyüklü, M., Yalçin, Ö., Başkurt, O.K., 2005. Exercise-induced oxidative stress leads hemolysis in sedentary but not trained humans. J. Appl. Physiol. 99, 1434–1441. https://doi.org/10.1152/japplphysiol.01392.2004.

Sheldon, B.C., Kruuk, L.E.B., Alberts, S.C., 2022. The expanding value of long-term studies of individuals in the wild. Nat. Ecol. Evol. 6, 1799–1801. https://doi.org/10.1038/s41559-022-01940-7.

Shopsowitz, K.E., Shih, A.W., 2021. How red blood cell quality is starting to carry its weight. Transfusion 61, 336–339. https://doi.org/10.1111/trf.16264.

Smith, S.M., Nager, R.G., Costantini, D., 2016. Meta-analysis indicates that oxidative stress is both a constraint on and a cost of growth. Ecol. Evol. 6, 2833–2842. https://doi.org/10.1002/ece3.2080.

Speakman, J.R., Garratt, M., 2014. Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model: prospects & overviews. BioEssays 36, 93–106. https://doi.org/10.1002/bies.201300108.

Speakman, J.R., Blount, J.D., Bronikowski, A.M., Buffenstein, R., Isaksson, C., Kirkwood, T.B.L., Monaghan, P., Ozanne, S.E., Beaulieu, M., Briga, M., Carr, S.K., Christensen, L.L., Cochemé, H.M., Cram, D.L., Dantzer, B., Harper, J.M., Jurk, D., King, A., Noguera, J.C., Salin, K., Sild, E., Simons, M.J.P., Smith, S., Stier, A., Tobler, M., Vitikainen, E., Peaker, M., Selman, C., 2015. Oxidative stress and life histories: unresolved issues and current needs. Ecol. Evol. 5, 5745–5757. https://doi.org/10.1002/ece3.1790.

Theil, P., Pedersen, L., Jensen, M., Yde, C., Knudsen, K., 2013. Blood sampling and hemolysis affect concentration of plasma metabolites. J. Anim. Sci. 90, 412–414. https://doi.org/10.2527/jas.53968.

Tomin, T., Bordag, N., Zügner, E., Al-Baghdadi, A., Schinagl, M., Birner-Gruenberger, R., Schittmayer, M., 2021. Blood plasma quality control by plasma glutathione status. Antioxidants 10, 864. https://doi.org/10.3390/antiox10060864.

Tsikas, D., 2017. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: analytical and biological challenges. Anal. Biochem. 524, 13–30. https://doi.org/10.1016/j.ab.2016.10.021.

Vogelaar, S.A., Posthuma, D., Boomsma, D., Kluft, C., 2002. Blood sample stability at room temperature for counting red and white blood cells and platelets. Vasc. Pharmacol. 39, 123–125. https://doi.org/10.1016/S1537-1891(02)00298-7.

Wiersma, P., Selman, C., Speakman, J.R., Verhulst, S., 2004. Birds sacrifice oxidative protection for reproduction. Proc. Biol. Sci. 271, S360–S363.

Zera, A.J., Harshman, L.G., 2001. The physiology of life history trade-offs in animals. Annu. Rev. Ecol. Evol. Syst. 32, 95–126. https://doi.org/10.1146/annurev.ecolsys.32.081501.114006.