Fluctuating asymmetry could be reliable proxy for oxidative stress in vertebrates

The study of fluctuating asymmetry (FA) in living organisms has produced contradictory results over the past few decades. Though the protocol for measuring FA is firmly established, the sources of FA remain unclear in many cases. Our goal is to study the relationship between FA and both the concentration of biomarkers of reactive oxygen species (ROS) and the body condition in a medium-sized mammal, the European wild boar (Sus scrofa). Using a Partial Least Squares regression (PLSr) we found a positive significant relationship between oxidative stress and FA but a negative relationship between oxidative stress and body condition of boar. Our results may suggest that FA can be used to assess the physiological cost associated with oxidative stress in mammals.

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Can Fluctuating Asymmetry be used as a proxy for oxidative stress in mammals?

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ABSTRACT

The study of fluctuating asymmetry (FA) in living organisms has produced contradictory results over the past few decades. Though the protocol for measuring FA is firmly established, the sources of FA remain unclear in many cases. Our goal is to study the relationship between FA and both the concentration of biomarkers of reactive oxygen species (ROS) and the body condition in a medium-sized mammal, the European wild boar (*Sus scrofa*). Using a Partial Least Squares regression (PLSr) we found a positive significant relationship between oxidative stress and FA but a negative relationship between oxidative stress and body condition of boar. Our results may suggest that FA can be used to assess the physiological cost associated with oxidative stress in mammals.

KEYWORDS: Ecological Indicators; Developmental Instability; Physiological Stress; *Sus scrofa*.

INTRODUCTION

For more than two decades, Thornill and Møller (1997) [50] defined Developmental stability (DS), as the ability of a genotype to undergo stable development of a phenotype under given environmental conditions, has been proposed as a proxy for health status in a broad range of live organisms [42], including plants [26], animal species [2] and the human beings [14]. Deviations from developmental stability (e.g., Developmental Instability, DI) arise from the disruptive effects of a wide range of environmental and genetic stresses, and these deviations are usually measured in terms of fluctuating asymmetry (FA, see [24, 40, 50]).

However, FA is not fully accepted by the scientific community since it has been detected in the absence of stress [42]. Moreover, the lack of knowledge about the factors that predict associations between FA and stress sometimes hamper its use as an ecological indicator [47]. Nevertheless, the number of studies showing how FA increases with environmental stress (e.g., food restriction) continues to grow every year [23].

In this work, we will explore whether oxidative stress is a source of developmental instability in a medium-sized mammal. The formation of reactive oxygen species (ROS) is associated with the pathology of animal and plant diseases, as well as natural aging of individuals [5, 28]. In fact, oxidative damage of DNA appears to be the overwhelmingly primary cause of mutation [15].

Organisms have developed enzymatic protection against ROS that includes several enzymes such as catalase (CAT), superoxide dismutases (Mn- and CuZn-SOD), glutathione (GSH) reductase (GR), selenium-dependent glutathione peroxidase (Se-GPX), and selenium independent GPX, keeping ROS and other toxic by-products of oxidative damage (e.g., aldehydes) at concentrations that are non-threatening to the cell [1, 28].

Some works show that decay in body condition produced by starvation is induced due to the production and accumulation of ROS triggering cell autophagy [17]. Other studies suggest that a wide array of compounds that act as environmental pollutants may propitiate health consequences for exposed mammals and fish by triggering an overproduction of ROS [18]. Even extrinsic perturbations of the host environment that cause abnormal exercise and fatigue in individuals can lead to oxidative stress [37]. There is a clear connection between ROS concentrations in the organism and environmental stress, and thus extreme starvation, high radiation exposure, environmental pollution, and traumatic and infectious diseases can increase ROS concentrations [27]. Hence, the assessment of ROS status is a suitable indicator of both biotic and abiotic environmental stress.

Though FA has been used for assessing biological stress in a broad range of aquatic [3, 30, 32] and terrestrial [4, 22, 31, 38] animal species, to date the relationship between ROS activity and FA has only been tested in humans [20].

One of the main advantages of measuring FA in large mammal populations is the time required by individuals to achieve their full size. This would provide sufficient time for symmetrical structures to express developmental instability in the case of stress, making it easy to measure FA. Typical structures for measuring FA in large mammals are antlers [43], jaws [45], and tusks [19].

In this work, we aim to contribute to the study of FA in terrestrial vertebrate organisms using the wild boar (*Sus scrofa*) as a study model. In this wild pig, both the maxillary and the mandible permanent canines are developed as tusks [34]. The opposing maxillary and mandibular tusks wear each other down, thereby maintaining the normal length and functional shape of the teeth. There is a lifelong presence of formative tissues at the apical end of all dental pieces, and thus the teeth exhibit continuous growth that is especially evident in the tusks [36]. Hence, tusks are susceptible to developmental instability and consequently show fluctuating asymmetry.

The use of a metric trait such as tusk width implies continuous variation that allows the detection of differences between sides, or departures from FA, only limited by measurement precision and accuracy [40]. Metric trait measurements can be directly tested for dependence of the absolute differences between the right and left sides (|R-L|) on overall size for each trait and the contribution of measurement error relative to FA.

ROS-induced damage to DNA or cell membranes may disrupt cell replication, presenting the possibility that individual differences in susceptibility to oxidative stress should be associated with FA [20].

In this work, we explore the relationships between wild boar oxidative status and both FA and body condition in a medium-sized mammal using a Partial Least Squares regression (PLSr). The positive relationships between oxidative status and tusk FA suggest that developmental instability is affected by oxidative stress in mammals. In the same line, the negative relationship between oxidative status and body condition confirms the importance of measuring body condition as a general indicator of health in wildlife populations.

MATERIALS & METHODS

Study area

The study area is located in the National Game Reserve "Ports de Tortosa i Beseit" (NGRPTB), north-eastern Spain (40º 48' 28" N, 0º 19' 17'' E). The NGRPTB is a limestone mountain massif of about 28,000 ha that shows a high level of orographic complexity, which results in a rugged and abrupt terrain formed by numerous canyons, ravines and steep slopes. About 28% of the surface is above 1000 m.a.s.l. The mean annual temperature in the reserve is 13.7ºC (min= 1.6ºC in December – February, max = 30ºC in July – August), while the mean annual accumulated rainfall is 697 mm (min = 536 in 2009, max = 889 in 2011). The vegetal stratum is characterized by a

136 typical Mediterranean forest dominated by *Quercus ilex* and *Pinus halepensis*
137 with dense scrublands of *Quercus coccifera*, *Pistacia lentiscus* and
138 *Chamaerops humilis*, among others. The average density of wild boar is 3
139 individuals/km^2 (estimate based on hunting bags for the whole reserve).

140 **Wild boar sampling and biometry**

141 Taking advantage of the regular game activities carried out in the NGRPTB,
142 63 hunter-harvested wild boar (30 females and 33 males) were collected
143 between May 2009 and February 2013. The sex of animals was determined
144 by observation of their sexual organs. Jaws were then removed from the
145 skull, labeled and stored in a cold box for transportation to our facilities at
146 the University (UAB). Rump fat (RF), measured using a metal rule (nearest
147 0.5 mm), was used as a proxy for wild boar body condition. Boars were then
148 dissected and 10 gr of spleen was collected and stored in individual plastic
149 bags and kept in a cold box (4ºC). Spleen samples were later frozen at -20°C
150 for the ROS analysis within the following 5 hours.

151 Using the jaws, age of boars was determined by the eruption of dentition
152 pattern [7]. For the calculation of the FA index soft tissues were removed
153 from fresh jaws before they were boiled in a 1% potassium hydroxide (KOH)
154 solution. Once cleaned and dry, basal width (medial view) of the right and
155 left tusks of each boar was measured twice by the same observer at different
156 times in order to minimize inter-observer variability [40]. Measurements for
157 one or both tusks were missing for some individuals, so these entries were
158 removed from the data pool resulting in a definitive number of 40 individuals

(19 females and 21 males). The data file layout followed the format recommended by Palmer (1994).

Oxidative stress assessment

Lipid peroxidation (TBARS), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and superoxide dismutase (SOD) concentration were estimated from spleen samples following specific procedures for each indicator. In brief, laboratory procedures were the following: five grams of spleen tissue were frozen in liquid nitrogen and stored at -80ºC for almost 30 days. Later, tissues were homogenized with an electrical homogenator (Miccra D-1 Art Moderne Labor Technik) in cool homogenization buffer (Tris-HCl 100 mM, EDTA 0.1 mM, Triton X-100 0.1 %, pH 7.8) in a 1:4 proportion (1 g tissue: 4 ml buffer). The sample was centrifuged at 14,000 rpm 4ºC for 30 minutes and supernatant stored at -80ºC until enzymatic determination. The activity of oxidative enzymes was estimated following specific procedures. TBARS (mmol MDA/mg) was estimated measuring the malondialdehyde (MDA) of the sample and those generated from lipid hydroperoxides by the hydrolytic conditions of the reaction [8]. MDA is a low-molecular-weight molecule formed via the decomposition of primary and secondary lipid peroxidation products. The aforementioned technique minimizes additional oxidation of the sample matrix that would overestimate lipid peroxidation [39]. SODs (U/mg) are enzymes that provide an important antioxidant defense in nearly all cells exposed to reactive oxygen species

182 generated by a cellular immune response. SOD catalyzes the dismutation of
183 superoxide into oxygen and hydrogen peroxide measured by the inhibition
184 degree of cytocrome C by this enzyme. The method followed for its
185 estimation was that proposed by [12]. The GPX (mU/mg) concentration, a
186 selenium-dependent protein that catalyzes the reaction of hydrogen
187 peroxides into water and alcohol, was determined by estimating NADPH
188 oxidation by the method proposed by [9]. The enzymatic activity of the GR
189 (mU/mg) was measured by the same mechanism following the method
190 described by [13]. On the other hand, CAT (U/mg) catalyzes the
191 decomposition of hydrogen peroxide produced in damaged tissues to water
192 and oxygen. CAT was estimated following the previously described method
193 [11]. Biochemical analyses were performed at the Laboratory of
194 Ecophysiology of the Estación Biológica de Doñana, Spain (EBD-CSIC) in a
195 multiplate reader Victor 3 Perkin Elmer, Massachussetts, USA. Concentrations
196 of the abovementioned enzymes will be used as a proxy for oxidative status
197 of individuals [29, 49].
198
199 **Statistical analysis**
200 The index used in the measurement of fluctuating asymmetry (FA) was FA1
201 according to [40]. This index provides an absolute (unsigned) measure of the
202 asymmetry. The FA1 index is easily and intuitively interpreted and gives a
203 direct indication of the level of asymmetry present within the sample for the
204 chosen trait. FA1 was chosen because it is easy to compute and represents

an unbiased estimator of the sample standard deviation. In addition, it can be directly subjected to the ANOVA testing procedure. According to [40], it is the most generally useful index for moderate to large sample sizes (e.g., 30 - 50) and is recommended where directional asymmetry and antisymmetry are absent and mainly where |R-L| is not dependent on overall size.

The analysis partially followed the step-by-step guide developed by [41]. The first steps of the guide emphasize the detection of outlier and aberrant individuals. The initial approach was a normality analysis of the difference between replicated measurement values (M2-M1) followed by a series of visual inspections of scatter plots and histograms of these replicate measurement differences by individual. This simple procedure provided an immediate indication of bad measurements and potential outlier data points. Once these points were detected, a Grubb's statistical test for outliers was performed. Once the potential outliers were confirmed and excluded, a graph plot of R-L versus the average length of the replicated measurements was used to confirm the nonexistence of any additional potential outlier.

The next step consisted of a test for between-sides differences due to measurement error and was carried out with a mixed model ANOVA of sides (fixed factor) * individuals (random factor). This test was used to determine the existence of directional asymmetry and the dependence of the side length differences on individuals. The analysis of asymmetry variation was justified because the between-sides variation was significantly greater than that expected due to measurement error (Sides * individuals, $F_{1, 79} = 15$, $P <$

0.001, $F_{1, 41} = 20.8$, $P < 0.001$ and $F_{1, 37} = 12.5$, $P < 0.001$ for the entire sample, females and males respectively). Between-sides variance was 10.8 % for females and 42.8 % for males. Thus, differences between replicated measurements were lower than the absolute differences between right and left sides of the trait.

A residual analysis confirmed the requirements of mixed models (e.g., linearity, homocedasticity and normality). Finally, the potential size dependence of FA was discarded by a regression test between trait size [(R+L)/2] and FA ($r = -0.011$ and $P = 0.778$ for the entire sample, $r = 0.019$ and $P = 0.184$ for females and $r = -0.027$ and $P = 0.970$ for males). In addition, a t-test was conducted to compare significant FA differences between males and females.

The relationship between oxidative stress, FA and body condition was evaluated by a Partial Least Squares regression approach (PLSr). This statistical tool is an extension of multiple regression analyses where associations are established with factors (e.g., combinations of dependent variables extracted from predictor variables that maximize the explained variance in the dependent variable). It is particularly useful when we need to predict a set of dependent variables from a (very) large set of independent variables (i.e., predictors). PLSr copes with multicollinearity better than generalized linear models [21]. The relative contribution of each variable to the derived factors was calculated by means of the square predictor weights. In our case the response variables were both the rump fat (RF) and the

fluctuating asymmetry, whereas the explanatory variables were the concentration of each biomarker of oxidative stress (i.e., TBARS, SOD, GPX, GR and CAT). The use of this approach minimizes the limitations derived from the use of a single biomarker of oxidative stress for describing the reasons for poor body condition of the individuals (high FA or low RF). The "plspm" library version 0.3.7 [44] of the R software version 3.1.1 [49] was used for these analyses.

RESULTS & DISCUSSION

Only one individual expressing aberrant levels of asymmetry was removed from the data pool. Our mixed ANOVA confirmed that R-L differences depended on the individuals validating the use of tusk width as a suitable trait for the calculation of the FA index. The same ANOVA test also confirmed the non-existence of Directional Asymmetry in our sample. In the same line, there was a lack of correlation between the |R-L| difference and the average trait size. Normality of residuals was also achieved.

The FA1 index for the trait selected was 0.31 for the entire sample, with slight but not significant ($t_{1, 38} = 0.87$, $P > 0.05$) differences between females (FA1 = 0.35) and males (FA1 = 0.27), representing a subtle 1.7 % of the trait size. Other works have shown typical levels of FA around 1% of trait size (Lens *et al.*, 2002). In addition, these FA values were independent of the age of animals ($r = 0.009$ $P > 0.05$).

The PLSr analysis provided a first factor based on the combination of the biomarkers of oxidative stress explaining 24 % of the variance of FA and body condition of wild boar. CAT was the most important biomarker explaining more than 54 % of the bloc describing oxidative stress, followed by TBARS with 42% and SOD with 1.4 % (Table 2). The best correlations between biomarkers of oxidative stress and the PLS Y's component (FA + RF), were reached by CAT (r = -0.68) followed by TBARS (r= -0.45). The rest of the biomarkers were poorly correlated (r = 0.0064 for GPX, r = -0.006 for GR and r = -0.075 for SOD).

The PLSr analysis showed different score signs for the two response variables selected (rump fat and fluctuating asymmetry), while three out of the five biomarkers of oxidative stress (TBARS, CAT and SOD) selected as explanatory variables contributed significantly to explaining the variance of the response variable group (loadings shown in Table 2). Weights of these three biomarkers presented the same sign as FA (higher oxidative stress in animals showing high FA) but the opposite sign to RF (higher ROS values in animals showing poor body condition). By plotting a circle of correlations we can take a visual glimpse of the relationships between the explanatory variables (ROS Biomarkers) and the response variables (FA and Rump Fat, Fig. 1). We case see how the factors clustered together are correlated positively whilst those factors distanced are not. When there is a degree of separation between factors of different axes it means a negative correlation between the factor in question and the rest of the variables.

Our graphic shows a positive correlation mainly between FA1 and CAT, but also between FA1, SOD and TBARS. On the other hand, we can see the Rump Fat opposing the rest of oxidative markers indicating the inverse relationship between body condition and oxidative stress.

When ROS production exceeds a tolerable threshold, the organism experiences oxidative stress and oxidative damage. The production of antioxidants and repair processes may constitute important allocations to somatic effort, and may be particularly relevant for species with low extrinsic mortality [16]. Because ROS are intrinsic costs of energy production itself, oxidative stress is a constraint on other expenditures, leading to a lower body condition of the individuals and induced damage to DNA. Lowered body condition and damaged DNA can break the fragile balance of developmental homeostasis that maintains the proper flow of development for the population.

The PLSr statistical model confirmed a significant positive relationship between ROS biomarkers and FA index and a negative relationship between ROS biomarkers and body condition of boars. Considering the established relationship between body condition and ROS, this result makes way for the use of FA as an indicator of physiological stress for wild boar populations given the general acceptance of rump fat concentration as a measure of body condition for ungulate species. Nonetheless, further research is in order to ensure a generalized conclusion.

As suggested by previous literature [20], and derived from the results shown in this work, we believe that a sound relationship between ROS and FA can be established. If we take into account the continuous growth of the tusks, this trait becomes considerably susceptible to the effects of developmental noise caused by any kind of stress, so we can conclude that the expression of levels of FA detected by our analyses reinforces the suggested relationship between FA and biotic or abiotic environmental stress.

CONCLUSIONS

Firstly, derived from our results, a sound relationship between ROS biomarkers and FA can be firmly established, and hence a relationship can also be established between oxidative stress and FA. Secondly, we can confirm our selected trait as suitable for the evaluation of the levels of FA within populations of wild boar. Finally, FA can be used to rapidly examine the status of wild boar populations and act as an early warning signal for the management of the hosting environment of the species.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing interests

The authors declare there are no competing interests.

Author Contributions

- Miguel Cánovas, analyzed the data, wrote the paper, prepared figures and/or tables.
- Gregorio Mentaberre, Encarna Casas, Nora Navarro-González and Santiago Lavín, performed the field sampling, prepared the jaws and reviewed drafts of the paper.
- Asta Tvarijonaviciute, Rafaela Cuenca and Ramón Soriguer, contributed reagents/materials/analysis tools, and, reviewed drafts of the paper.
- Emmanuel Serrano conceived and designed the experiment, analyzed the data, and reviewed drafts of the paper.

Conflicts of Interest

The author declares no conflict of interest.

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Table 1. Predictor weights of the Partial Least Squares regression (PLSr) analysis explaining the effect of several biomarkers of oxidative stress on FA and body condition (rump fat). Predictor weights represent the contribution of each explanatory variable to the PLSr model variance.

Predictor variable	Weight	% Variance explained
CAT	-0.74	54.76
TBARS	-0.65	42.25
SOD	-0.12	1.44

530

531 **Table 2.** Predictor and response loadings of the Partial Least Squares
532 regression (PLSr) analyses showing a positive correlation (same sign)
533 between FA and oxidative stress biomarkers and a negative correlation
534 (opposite sign) between rump fat and ROS biomarkers.

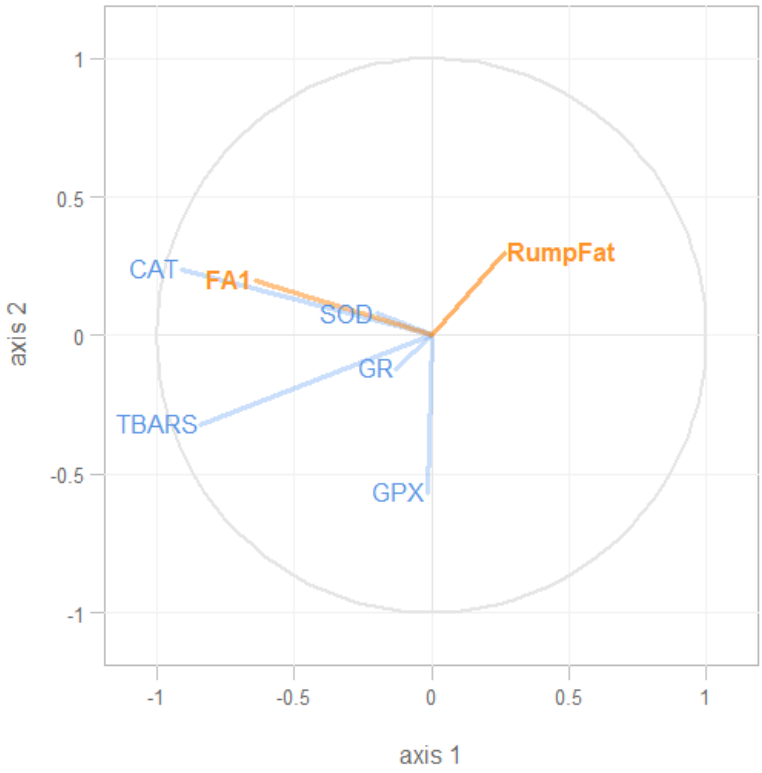
535

X's component	Loading	Response variable	Loading
CAT	-0.72	FA	-0.51
TBARS	-0.66	Rump Fat	0.21
SOD	-0.15		
GR	-0.10		
GPX	-0.01		

536

FIGURE CAPTION

Figure 1. Predictor and response loadings of the Partial Least Regression (PLSr) analyses showing positive correlation (same sign) between FA and oxidative stress biomarkers and negative correlation (opposed sign) between rump fat and ROS biomarkers.



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543 **FIGURE 1**