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Fluctuating asymmetry as a proxy for oxidative stress in wild boar

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ABSTRACT

The study of fluctuating asymmetry (FA) in living organisms has produced contradictory results over the past few decades of research. Though the protocol for measuring FA is firmly established, the sources of FA remain unclear in many cases. Our goal is to examine the relationship between FA and both the concentration of biomarkers of reactive oxygen species (ROS) and 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 (Stone–Geisser test) between oxidative stress and FA but a negative relationship between oxidative stress and body condition. Our results suggest that FA can be used to assess the physiological costs associated with oxidative stress in mammals.

Keywords: Ecological indicators; Developmental instability; Physiological stress; *Sus scrofa*.

Introduction

Developmental stability (DS), defined 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, including plants (Hagen et al., 2008), animals (Allenback, 2011) and human beings (Thornhill and Møller, 1997, DeLeon, 2007). Deviations from developmental stability (e.g., Developmental Instability, DI) arise from the effects of a wide range of environmental and genetic stresses and are usually measured in terms of fluctuating asymmetry (FA, see Graham et al., 2010). However, FA is not fully accepted by the scientific community for this purpose because it does not always respond to obvious stress (Floate and Coghlin, 2010). In fact, the concept of developmental stability is often elusive and low FA is not the unambiguous measure of well-being or good genes that some have claimed it to be (Rasmuson, 2002). Unfortunately, all of the previous mentioned factors hamper the use of FA as an ecological indicator and thus further studies assessing the integration of not only FA, but also other health indicators, are needed for further progress. Despite this doubt, and probably due to the ease of calculating FA, the number of studies on the uses of this biomarker continues to grow (González et al., 2014).

Another biomarker of both biotic and abiotic environmental stress is the oxidative status of organisms. The formation of reactive oxygen species (ROS, including O_2- ,H_2O_2, and OH), is associated with the pathology of animal diseases, as well as the natural aging of individuals (Dalle-Donne et al., 2006). Organisms have developed enzymatic protection against ROS including catalase (CAT), superoxide dismutases (Mn- and CuZn-SOD), glutathione reductase (GR), selenium-dependent glutathione peroxidase (Se-GPX), and selenium-independent GPX, which maintain ROS and other toxic by-products of oxidative damage (e.g., aldehydes) at concentrations that are non-threatening to the cell (Ahmad, 1995, Held, 2012). Some work shows that decay in body condition produced by starvation is induced by the p roduction and accumulation of ROS triggering cell autophagy (Elazar et al., 2007). 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 (Farmen et al., 2010). 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 (Halliwell and Cross, 1995). Controversially, the relationship between ROS activity and FA has only been tested in humans (Gangestad et al., 2010).

In this work, we aim to study FA in the wild boar (*Sus scrofa*) as a study model. 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). 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 jaws (Serrano et al., 2008) and tusks (Modi et al., 1987).

In wild boar, both the maxillary and the mandible's permanent canines are developed as tusks. There is a lifelong presence of formative tissues at the apical end of all dental pieces, and thus they are susceptible to developmental instability and consequently show fluctuating asymmetry (Palmer, 1994; Palmer and Strobeck, 2003). 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 (Palmer, 1994). 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 (Gangestad et al., 2010). Hence, a negative relationship between body condition and oxidative status will be in line with previous research (Sorensen et al., 2006), whereas the positive relationship to FA would suggest a link between developmental instability and oxidative stress in mammals.

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 characterized by a typical Mediterranean forest with dense scrublands.

Taking advantage of the regular game activities carried out in the NGRPTB, 63 hunter-harvested wild boar (30 females and 33 males) were collected between May 2009 and February 2013. The sex of animals was

determined by observation of their sexual organs. Jaws were then removed from the skull, labeled and stored in a cold box for transportation to our facilities at the University (UAB). Rump fat (RF), measured using a metal rule (nearest 0.5 mm), was used as a proxy for wild boar body condition. Boars were then dissected and 10 g of spleen was collected and stored in individual plastic bags and kept in a cold box (4ºC). Spleen samples were then frozen at -20°C for the ROS analysis within the following 5 hours.

Using the jaws, age of boars was determined by the eruption of dentition pattern. For the calculation of the FA index soft tissues were removed from fresh jaws before they were boiled in a 1% potassium hydroxide (KOH) solution. Once cleaned and dry, basal width (medial view, Fig. 1) of the right and left tusks of each boar was measured twice with an electronic digital caliper (IP54, iGaging EZ®, accuracy: 0.02 mm). Measurements were taken by the same observer (ES) at different times in order to minimize inter-observer variability (Palmer, 1994). Measurements for one or both tusks were not possible in 23 individuals given that their dental pieces were damaged during transportation. Hence, these individuals were excluded from the analysis.

Lipid peroxidation (TBARS), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and superoxide dismutase (SOD) concentrations 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. Tissues were then 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 at 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. MDA is a low-molecular-weight molecule formed via the decomposition of primary and secondary lipid peroxidation products. The aforementioned technique minimizes the additional oxidation of the sample matrix that would overestimate lipid peroxidation (Monaghan et al., 2009). SODs (U/mg) are enzymes that provide an important antioxidant defense in nearly all cells exposed to reactive oxygen species generated by a cellular immune response. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide measured by the inhibition degree of cytochrome C by this enzyme. The method followed for

its estimation was that proposed by Cord and Fridovich (1969). The GPX (mU/mg) concentration, a selenium-dependent protein that catalyzes the reaction of hydrogen peroxides into water and alcohol, was determined by estimating NADPH oxidation by the method proposed by Carmagnor and Sinet (1983). The enzymatic activity of the GR (mU/mg) was measured by the same mechanism following the method described by Cribb et al. (1989). On the other hand, CAT (U/mg) catalyzes the decomposition of hydrogen peroxide produced in damaged tissues to water and oxygen. CAT was estimated following a previously described method (Cohen and Somerson, 1969). Biochemical analyses were performed at the Laboratory of Ecophysiology of the Estación Biológica de Doñana, Spain (EBD-CSIC) in a multiplate reader Victor 3 Perkin Elmer, Massachusetts, USA. Concentrations of the abovementioned enzymes were used as a proxy for oxidative status of individuals.

The index used in the measurement of fluctuating asymmetry (FA) was FA1 according to Palmer (1994), defined as:

FA1 = mean |Right trait size – Left trait size|

This index provides an absolute (unsigned) measure of the asymmetry. The FA1 index is easily and intuitively interpreted and gives a direct indication of the level of asymmetry present within the sample for the chosen trait. FA1 was chosen because it can be directly subjected to the ANOVA testing procedure, is adequate for moderate sample sizes and is not dependent on overall size.

The analysis partially followed the step-by-step guide developed by Palmer and Strobeck (2003). The analysis of asymmetry variation was justified because the between-sides variance component was greater than zero, after removing the effect of measuring 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). The between-sides variance component for females was 10.8% and 42.8% for males, both greater than the measurement error (lower than 4.5% in both males and females). A residual analysis confirmed the requirements of mixed models (e.g., linearity, homoscedasticity 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).

The relationship between oxidative stress, FA and body condition was evaluated by a Partial Least Squares regression (PLSr). This statistical tool is an extension of multiple regression analyses where associations

are established with factors, also called components or latent vectors (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 (Carrascal et al., 2009). In our case, the response variables were both the rump fat (RF) and the fluctuating asymmetry, and 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 in individuals (high FA or low RF). The "plspm" library version 0.3.7 (Sánchez and Trinchera, 2007) of the R software version 3.1.2 (R Development Core Team, 2015) was used for these analyses.

Only one individual expressing aberrant levels of asymmetry was removed from the data pool. Our mixed-model 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. Likewise, there was a lack of correlation between |R-L| and the average trait size. Normality of residuals was also achieved.

The average FA1 index for the trait selected was 0.31 for the entire sample, with slight but not significant (t-test$_{1, 38}$ = 0.87, P = 0.43) differences between females (FA1 = 0.35) and males (FA1 = 0.27), representing a subtle 1.7% difference in 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 = 0.35).

The PLSr analysis provided a first factor based on the combination of the biomarkers of oxidative stress explaining 25.8% of the variance of FA and body condition of wild boar. CAT was the most important biomarker explaining 67.7% (square of the PLSr weight value, Table 1) of the PLSr component describing oxidative stress, followed by TBARS with 30.7%. GPX, SOD and GR showed a low contribution explaining less that 1% of the X's component. The best correlations between biomarkers of oxidative stress and the PLS Y's component (FA + Body condition) were reached by CAT (r = - 0.67) followed by TBARS (r= - 0.45). The rest of the biomarkers were poorly correlated (r = 0.074 for GPX, r = -0.068 for SOD and r = -0.009 for GR).

The PLSr analysis showed different score signs for the two response variables selected (body condition and fluctuating asymmetry), while two out of the five biomarkers of oxidative stress (TBARS, CAT) selected as explanatory variables contributed significantly to explaining the variance of the response variable group (loadings shown in Table 1). Weights of these two biomarkers presented the same sign as FA (lower oxidative stress in animals showing low FA) but the opposite sign of RF (low ROS values in animals showing good body condition, Fig. 2).

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 (Dowling and Simmons, 2009). Because ROS are intrinsic costs of energy production itself, oxidative stress is a constraint on other expenditures, leading to a lower individual body condition 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 selected ROS biomarkers and FA index and a negative relationship between ROS biomarkers and body condition in 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 necessary to ensure a generalizable conclusion.

As suggested by Gangestad et al., (2010), 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, and thus 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.

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.

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Table 1. Predictor loadings, weights and the variable importance for projection VIP of the Partial Least Squares regression (PLSr) between several biomarkers of oxidative stress (X's component): catalase (CAT), lipid peroxidation (TBARS), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR), and the Y's component describing body condition (rump fat thickness) and fluctuating asymmetry (AF) in wild boars. Predictor weights represent the contribution of each explanatory variable to the PLSr model variance. The VIP is a measure of explanatory power of Y; those predictors with a VIP > 1 are considered the most relevant to the construction of the Y-component.

Predictor variable	Loadings	Weight	VIP
PLS-R X's component describing oxidative stress			
CAT	-0.757	-0.822	1.839
TBARS	-0.654	-0.554	1.240
GPX	-0.052	0.091	0.204
SOD	-0.121	-0.084	0.189
GR	-0.065	0.012	0.027
PLS-R Y's component describing fluctuating asymmetry and body condition			
FA	-0.555	-0.939	-
Body condition	0.221	0.505	-

274

275 **FIGURE CAPTION**

276 **Figure 1**. For the calculation of fluctuating asymmetry in wild boar, basal width in medial view of the right and left
277 definitive tusks (black arrow), was measured with a digital caliper (0.02 mm accuracy).

278 **Figure 2.** Positive relationship between oxidative stress biomarkers and fluctuating asymmetry (FA) of tusks and
279 negative correlation (opposed sign) between body condition (rump fat thickness) and specific ROS biomarkers
280 (CAT: catalase and TBARS: lipid peroxidation). This plot represents the PLSr model shown in Table 1. Arrow
281 direction indicates either an increase or a decrease in the component value, and arrow thickness directly indicates
282 the weight of the component. ROS biomarkers explained <5% of the PLSr X component (e.g., SOD: superoxide
283 dismutase, GPX: glutathione peroxidase and GR: glutathione reductase) and were therefore excluded from the
284 plot.

285

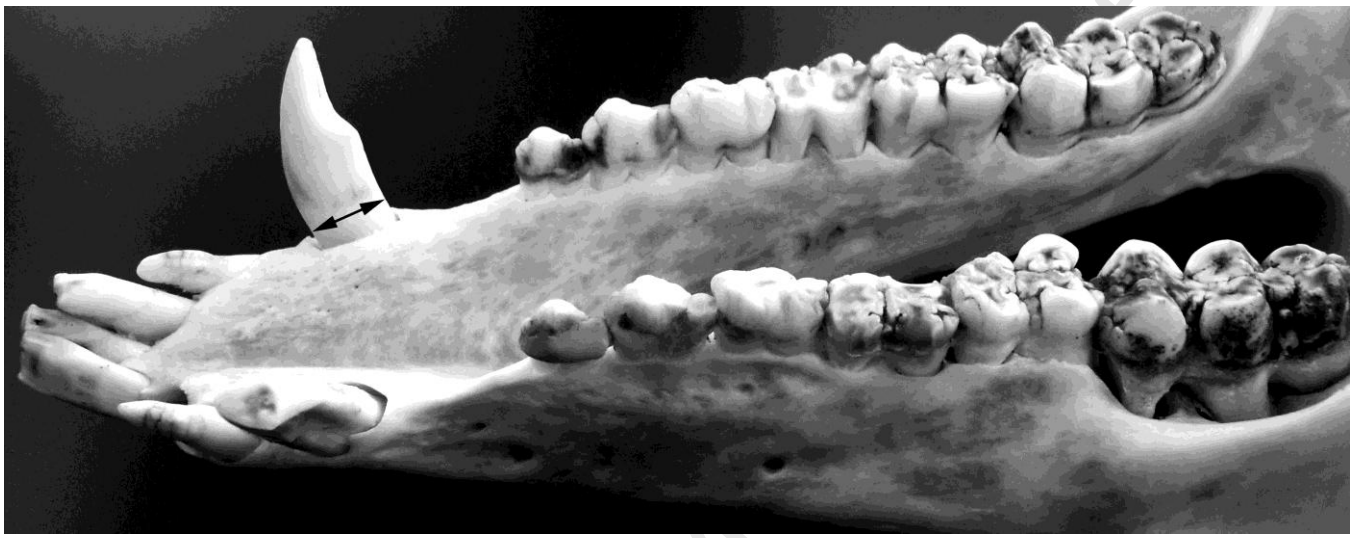

FIGURE 1

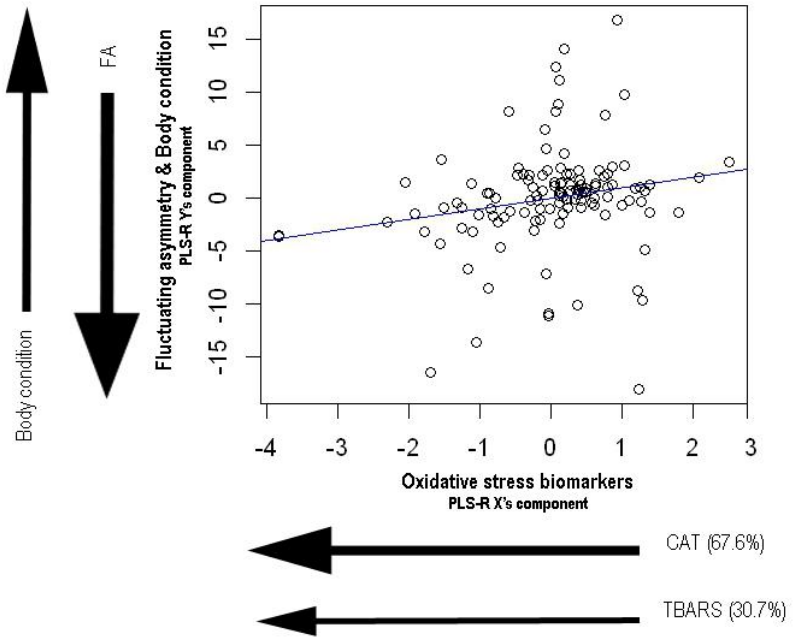

FIGURE 2