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Short Communication

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.

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 production 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

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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 European wild boar (*Sus scrofa*) as a study model. We explore the relationships between wild boar oxidative status and both FA and body condition 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 (40° 48′ 28″ N, 0° 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 ruler (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 (4 °C). 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 −80 °C 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 4 °C for 30 minutes and supernatant stored at −80 °C 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 Carmagnol et al. (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

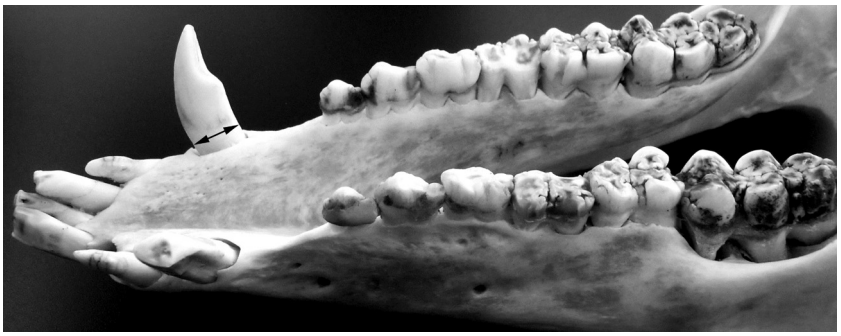

Fig. 1. For the calculation of fluctuating asymmetry in wild boar, basal width in medial view of the right and left definitive tusks (black arrow), was measured with a digital caliper (0.02 mm accuracy).