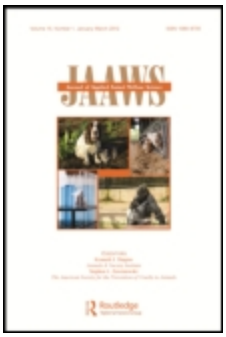

# Assessment of welfare in wild boars by analysis of hormonal and oxidative stress markers



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**Abstract**

This study demonstrated that it is possible to use stress level markers in wild animals. Reactive oxygen metabolites (ROMs), the antioxidant barrier, i.e. the biological antioxidant potential (BAP) and the antioxidative power (OXY adsorbent), and cortisol were measured in freely ranging wild boars to collect data to improve wildlife management. In two areas (C and D) of a State Forest in the Campania Region, 42 freely ranging, managed wild boars were captured with a corral trap, and blood samples were collected. The sampled wild boars were divided by age (>1 year old and <1 year old) and sex (male and female). Animals in the D area (both males and females) were subject to significantly greater oxidative stress than those living in the C area. These results may be explained by a higher population density in the D than C area. Furthermore, data showed that in D area the stress parameters were significantly higher in males compared to females and that the same trend was put in evidence considering the class of age (>1 year old respect to <1 year old).

**Keywords:** Wild boar; wildlife welfare; oxidative stress markers; cortisol; free-range breeding.

## Background

Wild boar (*Sus scrofa*) numbers have continuously increased throughout Europe (Amici et al., 2015; Massei et al., 2014; Ramanzin et al., 2010), and numerous studies have demonstrated an increasing conflict between wild boar and human activity, principally agriculture (Kruuse et al., 2016; Hua et al., 2016; Lebocký & Petráš, 2015; Laznik &Trdan, 2014; Ficetola et al., 2014). Few studies have been conducted on the effect of the population density on the welfare of these *Suidae* in the disturbed areas. Ecological, health and welfare issues are usually considered together in the management of wild fauna.

The health and welfare problems faced by wild animals are major focuses of the European community (Esposito & Amici, 2009). A major problem is the need to evaluate the welfare of animals in the dual context of biodiversity conservation and their commercial role in the World Trade Organization (Brückner, 2014). In the latter case, the maintenance of wild game in captivity presents the same welfare problems that are common to domestic species (Ranade et al., 2014). Welfare monitoring represents one of the instruments used to measure the correct management procedures for wild fauna. Wild boar is a possible wild animal model to evaluate stress factors involved in animal welfare.

Vertebrates cope with different environmental stimuli by initiating a stress response. The activation of the hypothalamic–pituitary–adrenal (HPA) axis and the resultant secretion of glucocorticoid hormones (primarily cortisol) are unavoidable in this response (Sheriff et al., 2011).

Oxidation is a ubiquitous phenomenon on earth, and all organisms are thus exposed to its damaging effect on cells. The increase in the oxidation state of biomolecules mediated by free radicals produced by cellular respiration or by exogenous factors can negatively impact the function of those biomolecules and ultimately leads to cellular deterioration and even to cell death (Finkel & Holbrook, 2000; Tafuri et al., 2015). Several factors may affect the oxidative status of an individual, and immune response (Klebanoff & Clark, 1978; Costantini & Dell'Omo, 2006), sexual hormones (Zhu et al., 1997), stress hormones (Ohtsuka et al., 1998), thermal conditions (Flanagan et al., 1998), diet (Sohal & Weindruch, 1996) and/or growth (Costantini et al., 2006) may affect the balance between pro-oxidants and antioxidants, increasing oxidative stress (Finkel & Holbrook, 2000 ). To counteract these effects, the body can rely on an antioxidant barrier, which is composed of both external (carotenoids, ascorbate, vitamin E, etc.) and internal (GSH, proteins, bilirubin, uric acid, etc.) substances. Each of these components blocks the potential damaging action of free radicals, and their reactivity is precisely linked to the particular shortage

of these small negatively charged particles. Any insult against this barrier allows free radicals to attack and damage cell structures, setting the stage for lesions typical of oxidative stress and all their dangerous consequences (premature aging, disease, etc.) (Ciani et al., 2015).

The present study involved two groups of wild boars maintained in a semi-free condition in a State Forest in the Campania region and aimed to assess the boars' blood levels of cortisol and some markers of oxidative stress. The pro-oxidant state was evaluated by measurements of ROMs, and the antioxidant barrier was evaluated by the BAP test and the OXY adsorbent test. The comparison of the parameters involved in stress processes can provide information about the welfare of an animal that is considered a pest by humans but that is part of the Italian fauna.

## Methods

### Study area and habitat

The study area, called "Cerreta Cognole", is classified as a State Forest in the Campania region. It is located between 40°15'41.64"N - 15°39'10.90"E and 40°13'24.49"N - 15°38'29.66"E from north to south and between 40°14'39.23"N - 15°39'52.53"E, and 40°14'57.32"N - 15°37'13.38"E from east to west. In total, the area, which is part of the "Cilento, Vallo di Diano e Alburni" National Park, covers 823 hectares.

The altitude ranges between 550 and 1,100 meters above sea level; the temperatures vary from -2°C to 28°C, with an average annual precipitation of 800-900 mm/yr.

Approximately 90% of the forest is covered with the dominant tree species, Austrian oak (*Quercus cerris*), of which 70% is mature forest and 20% is coppice (immature or degraded forest), while the remaining percentage is accounted for by mixed coppice Mediterranean forests, namely, deciduous species (*Fraxinus ornus*, *Ostrya carpinifolia*, *Acer spp.*), coniferous species (*Pinus halepensis*, *Pinus domesticus*, *Pinus pinaster*), chestnuts (*Castanea sativa*) and scrub (3%). A very small area (0.19%) is covered by pasture. The entire perimeter of the State Forest is completely fenced (27 km), and the whole area is subdivided into four 200-ha fenced zones (A, B, C, D), each of which has internal water sources (streams and canals).

This study was performed in two areas, area C, which is 222.3 hectares, and area D, which is 217.7 hectares. Data from 2011 indicated that area C contained 22 wild boars per 100 hectares, and area D

contained 43 wild boars per 100 hectares (Esposito et al., 2011). In each area, two adequate capture corrals are located as follows: C1 (40°14'16.12"N - 15°38'24.28"E), C2 (40°14'9.58"N - 15°37'57.15"E), D1 (40°14'38.42"N - 15°38'37.98"E), and D2 (40°15'0.88"N - 15°38'16.05"E). The wild boars freely feed, although the corrals, in addition to aiding in capturing the animals to sell, are used for food supplementation during specific time of the year (winter-summer).

**Animals**

The study was carried out with 42 freely ranging, managed wild boars that were captured during two different periods, during the spring season, in four corrals inside the fenced areas (C and D) of a public wild animal breeding area. All captured wild boars were classified according to age (>1 year and <1 year) and two sexes (male and female).

To ensure better welfare conditions, the captured wild boars were maintained inside the corral for a minimum of 7 hours and a maximum of 10 hours, starting from one hour after the traps were set (sunset) to the time the boars were captured (sunrise).

The presence of veterinary staff ensured the appropriate handling of the wild animals and guaranteed that the investigation was carried out in accordance with the EU Directive 2010/63/EU and the guidelines of the Animal Ethics Committee of the University of Naples Federico II.

**Blood sampling**

From the corral, each animal entered a wooden chute that led to a containment cage. Once inside the cage, each subject was held by the throat with a dogcatcher's hook. Blood samples were drawn from the jugular vein by a Veno Jet tube (Terumo, Leuven, Belgium) with a multi sample needle 20-G x 1.5", according to good veterinary practice. After the blood sample was collected, each wild boar was marked with an ear brand, and then, the cage was weighed both with and without the animal, and the difference between the two weights established the animal's weight. The animal was released at the end of the procedure. The blood collection was performed early in the morning. Samples were transported on icepacks to our laboratory within 4 hours of collection. After refrigeration at 4°C, sera were recovered by gentle centrifugation (800 RPM for 5 min at 4°C). Sera with a hemoglobin content <0.3 mg/ml were considered suitable for further tests and labeled, before being stored at -20°C.

**Sample analysis**

The sera samples were used for the determination of ROMs, the BAP test, the OXY adsorbent test, and cortisol levels.

*Measurement of ROMs, BAP test and OXY adsorbent test*

The ROMs test determines the level of oxidative stress by measuring the amount of organic hydroperoxide (ROOH) that oxidizes N,N-diethyl-p-phenylenediamine (Alberti et al., 2000; Trotti et al., 2002; Brambilla et al., 2003, Tafuri et al., 2018). The phenomenon is associated with the progressive and gradual colour change to a pink reaction mixture, which was initially colourless. In the d-ROMs test, ROMs of a biological sample are able to generate alkoxyl and peroxyl radicals, according to Fenton's reaction. Such radicals are able to oxide an alkyl-substituted aromatic amine that is solubilized in a chromogenic mixture, thus producing a pink-coloured derivative, which is photometrically quantified at 505 nm. The intensity of the developed colour is directly proportional to the concentration of ROMs, according to Lambert-Beer's law, and is expressed as Carratelli units (1 CARR U = 0.08 mg hydrogen peroxide/dL) (Costantino et al., 2009).

The antioxidant barrier was evaluated using the BAP test and the OXY adsorbent test (Tafuri et al., 2018). In the BAP (Biological Antioxidant Potential) test, adding a sample of plasma to a dye solution, obtained as a mixture of ferric chloride with a derived thiocyanate solution, a discoloration is caused. The intensity of this discoloration is determined photometrically by using a wave- length of 505 nm and it results proportional to the ability of plasma to reduce ferric ions. The results obtained are evaluated as µmol/L or reduced ferric ions.

By OXY-absorbent test was measured the ability of plasma to counteract the massive oxidation induced by a solution of hypochlorous acid (HClO), a powerful and physiological oxidant able to mimic situations that occur in vivo. Unreactive HClO radicals further react with the chromogen solution of N,N-diethyl-p-phenylenediamine and produce a colored complex, which is measured at 505 nm. The results were expressed as µmol HClO/L.

In the BAP test the antioxidant potential is evaluated as ferric reducing power, while in the OXY-adsorbent test is measured as ability of antioxidant barrier to oppose the massive oxidant action of HClO.

The oxidative stress kits were purchased from Diacron International, Grosseto, Italy.

*Measurement of cortisol*

Duplicate sera samples were tested for cortisol using a chemiluminescent enzyme immunoassay (Immulite 1000 Assay System, Siemens Healthcare GmbH, Erlangen, Germany) under well-controlled conditions and according to the manufacturer's instructions.

**Statistical analyses**

Van der Waerden's analysis of variance (ANOVA) was used to test the differences in reactive oxygen species (free radicals and non-radical species), as measured by ROMs, BAP, OXY, and serum cortisol between the areas of capture, sex and age of the sampled wild boars.

Depending on the data distribution, differences belonging to a specific category (intra sex and age) were explored by using an unpaired Student's t test. The percentage of wild boars captured in the different areas was tested by the Chi-square test. Significance was set at $P \leq 0.01$ and $P \leq 0.05$.

All statistical analyses were performed using a program for statistical analysis (JMP® 8.0 SAS Institute Inc.).

**Results**

Table 1 indicates the numbers of captured wild boars in areas D and C divided in age and sex classes. The male and female weights of the over 1 were significantly higher in D respect to C area ($P<0.01$, $P<0.05$ respectively), while there was no significant difference according to sex and age less than one year old (Table 1).

Data showed that regardless of sex, the boars in the D area were subject to significantly ($P<0.01$) greater production of ROMs than those living in the C area (Table 2). BAP values in both groups did not show any significant difference, while a significant difference of OXY was detected between the animals of the C and D areas. As shown in Table 2, total males had an antioxidant barrier significantly lower respect to total females ($P<0.01$); moreover considering the ROMs values, the relationship is inverted. The cortisol values were higher in area D respect C and in total males respect total females ($P<0.01$) (Table 2).

The results obtained between C and D areas and between males and females were confirmed also when the groups of animals are divided by gender and age, despite the shortness of the sample. The results are reported in Table 3. Cortisol was significantly higher in all animals in D area ($P<0.01$, in females <1 year old $P<0.05$). BAP was higher in animals in the C area and was significantly different in females of both

ages (P <0.01). The other oxidative stress parameters were higher in animals of D area; in particular, the ROMs in younger females than one year old were significantly higher in this area than in C area.

**Discussion**

This study showed that it is possible to use stress level markers in wild animals. In particular ROMs and the antioxidant barrier, i.e. the biological antioxidant potential (BAP) and the antioxidative power (OXY adsorbent, and cortisol were measured in freely ranging wild boars to collect data to improve wildlife management. The number of subjects was not consistent between the two study areas, but this is very common for studies with wild or semi-wild animals. Despite this limitation, the present study performed on wild boars revealed different stress and oxidative stress responses in the animals captured in the two survey areas, placed at "Cilento, Vallo di Diano e Alburni" National Park of Campania Region.

A state of oxidative stress is not an easy condition to identify in an absolute manner since it depends on the balance established between the prooxidants and antioxidants species (Esposito et al., 2017, Tafuri et al., 2018). Oxidative stress is a health risk factor involved in aging and in several diseases, in humans and/or in animals. However, if it is monitored over time, it can be a valid and predictive approach to health status.

The results obtained in this study suggest that the animals living in area D are more stressed than those in area C and that males are more subject to stress than females. These results may be explained first by the different population densities in areas D and C, with 33 wild boars/100 ha *vs* 9 wild boars/100 ha, respectively, and second by different physiological responses typical of gender that are closely linked to behavioral manifestations.

It is not easy to establish a condition of oxidative stress, so our results do not agree with another study carried out in swine and wild boars by Brambilla et al., (2003), where the absolute sera values of ROMs were lower than those described in our study. The differences found in the two studies can be explained by the different environmental and nutritional situations of the animals used in the two studies.

Given the different stress reactions according to sex and age in the two survey areas, it is possible to hypothesize that the conditions in the D area are more stressful, which is supported by the higher cortisol values in all animals in that area, with an even stronger response in males. When oxidative stress occurs, it is not enough to consider only the pro-oxidizing state (ROMs), but above all, it is critical to consider the

antioxidant state (BAP and OXY), which evaluates the ability of the antioxidant barrier to oppose the action of free radicals. The greater response of males older than one year old indicates that there could be an intraspecific competition in this class of animal that is more evident in the males of the D area than in those of the C area, due to a greater number of subjects. This competitive condition would more quickly trigger the metabolic processes that lead to increases in free radical production and the use of antioxidant components (Tafuri et al., 2016).

## Conclusions

This study demonstrated that it is possible to use different measurements of stress levels in wild animals. In a previous trial, we demonstrated that the cortisol level is a stress indicator in hares (*Lepus europaeus*) but that only one parameter is not sufficient to evaluate a response in a wild animal that is subjected to many variables (Esposito et al., 2017). The evaluation of both pro-oxidative and antioxidative states can help us to follow the metabolic responses of wild animals and improve wildlife management.

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Table 1. Number of wild boars (n), age and sex classes (>1 year old and <1 year old, M and F), live body weight (LBW kg) and standard deviation (SD) into two survey areas (C and D).

| | | Area C | | Area D | |
|---|---|---|---|---|---|
| Class of age | Sex | n. | LBW kg±SD | n. | LBW kg±SD |
| >1 year old | M | 3 | 69,67±1,53* | 4 | 80,50±3,70* |
| >1 year old | F | 2 | 61,50±3,54** | 14 | 67,93±5,43** |
| <1 year old | M | 2 | 39,50±2,12 | 5 | 43,20±2,39 |
| <1 year old | F | 2 | 37,50±2,12 | 10 | 40,40±4,50 |
| Total | | 9 | | 33 | |

*P < 0.01; **P<0.05

Table 2. Number of wild boars (M+F) in both areas of investigation (C and D) and all boars examined for different stress parameters (Cortisol, ROMs, BAP and OXY).

| | n | Cortisol (µg/dl) | ROMs (UCarr$^{+}$) | BAP (µmol/L) | OXY (mmol/L) |
|---|---|---|---|---|---|
| M+F C | 9 | 26.46±2.30* | 379.23±127.89* | 4107.42±777.16 | 312.38±62.88* |
| M+F D | 33 | 28.97±2.97* | 535.70±83.90* | 3736.74±829.92 | 356.92±65.89* |
| Total M | 14 | 29.98±2.73* | 449.33±106.30* | 3247.93±507.57* | 317.08±54.41* |
| Total F | 28 | 27.65±2.87* | 528.59±109.50* | 4100.29±813.13* | 362.53±68.54* |

*P <0.01

+ = 1UCarr corresponds to 0.08 mg hydroperoxide/100 mL $H_2O_2$

Table 3. Different stress parameters (Cortisol, ROMs, BAP and OXY) measured in animals (M and F) of different ages (>1year old and <1year old) in the two study areas (C and D).

| | M >1 year old | | F >1 year old | | M <1 year old | | F <1 year old | |
|---|---|---|---|---|---|---|---|---|
| | | | | Cortisol | | | | |
| Area | N | (µg/dl) | n | (µg/dl) | n | (µg/dl) | n | (µg/dl) |
| C | 3 | 29.13±0.28* | 2 | 25.10±0.17* | 2 | 26.51±0.66* | 2 | 23.77±1.44** |
| D | 4 | 33.76±0.68* | 14 | 28.83±2.72* | 5 | 28.86±1.16* | 10 | 27.29±2.58** |
| | | | | ROMs | | | | |
| | N | (UCarr) | n | (UCarr) | n | (UCarr) | n | (UCarr) |
| C | 3 | 383.84±122.24 | 2 | 367.92±196.07 | 2 | 384.50±246.44 | 2 | 378.38±37.56** |
| D | 4 | 520.28±55.28 | 14 | 607.82±37.87 | 5 | 457.81±36.53 | 10 | 479.85±79.45** |
| | | | | BAP | | | | |
| | N | (µmol/L) | n | (µmol/L) | n | (µmol/L) | n | (µmol/L) |
| C | 3 | 3817.45±482.98 | 2 | 4858.23±70.57* | 2 | 3081.86±85.67 | 2 | 4817.14±8.41* |
| D | 4 | 3301.14±459.94 | 14 | 4012.74±793.93* | 5 | 2930.08±417.11 | 10 | 3927.90±886.42* |
| | | | | OXY | | | | |
| | N | (mmol/L) | n | (mmol/L) | n | (mmol/L) | n | (mmol/L) |
| C | 3 | 293.95±40.07 | 2 | 342.79±57.23 | 2 | 238.81±24.96* | 2 | 383.20±28.31 |
| D | 4 | 320.20±43.91 | 14 | 350.48±66.12 | 5 | 359.76±38.29* | 10 | 379.20±81.29 |

*P< 0.01; **P <0.05