Seasonality of reproduction in wild boar (Sus scrofa) assessed by fecal and plasmatic steroids

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Abstract

The collection of biological samples through non-invasive techniques represents one way of monitoring *in vivo* physiological changes associated with reproductive activity. Such techniques are particularly important for the study of animal species in the wild.

The goals of this study were 1) to evaluate fecal progestogen (P), estrogen (E), and androgen (A) by means of radioimmunoassays, in male and female wild boars culled in the Piedmont, Italy area; 2) to compare them with plasmatic concentrations and the animals' reproductive status; and 3) to assess variations in reproductive seasonality between two populations of wild boars living in a mountainous vs. a plain habitat in Piedmont.

The results demonstrate a positive correlation between fecal and plasmatic steroid concentrations *(r* = 0.46, 0.58, and 0.45 for plasma P_4 and P, E_2 and E, and T and A; $P < 0.05$). Moreover, high fecal levels of both P and E (>170 ng/g and >100 pg/g respectively) were found in 70.6% of pregnant sows and in none of the non-pregnant animals, thus supporting the use of this technique for detecting pregnancy status in wild boar.

Similar birth patterns were displayed by the mountain and plain populations, but births peaked significantly only in the mountain population, in the spring (46%, $P < 0.05$, vs. other seasons). A corresponding autumnal peak of plasma testosterone concentrations in males was displayed only by the mountain population (7.4 vs. < 2.0 ng/mL in the other seasons, $P < 0.05$).

The correlation between fecal and plasmatic steroid concentrations obtained in this study supports the applicability of this non-invasive sampling technique for monitoring reproductive status in wild boar, thus enabling a more informed and correct management of the species.

Keywords: Wild boar; *Sus scrofa;* Reproductive seasonality; Fecal sex steroids; Plasma sex steroids

1. Introduction

The wild boar *(Sus scrofa),* an ungulate with a high reproductive capacity, is the species from which domestic pig breeds have been derived. Whereas the domestic pig is known to reproduce throughout the year, the wild boar is a seasonal polyestrous breeder with two reproductive periods. The most important period is characterized by oestrous cycles from November to March, and the secondary period presents mating in the spring [1,2]. Wild boars have colonized nearly the entire Piedmont region in northeastern Italy [3-6]. The causes of this population increase in recent decades are mainly a combination of ecological and environmental factors, such as the depopulation of rural areas, changes in agricultural practices and environment use, lack of predators, and climatic changes offering food and shelter to the animals [7-9]. Moreover, it is possible that animals derived from the crossbreeding of domestic swine and wild boars from Eastern Europe have been illegally introduced into the environment (in order to support human activities like hunting), and have thus favoured this demographic boom and affected genetic variation [10-14].

Through an understanding of wild boar reproductive periods, oestrus cycles and reproductive success rates, it will be possible to better manage the species population. In fact, only a careful analysis of the reproductive activities will lead to an understanding of the population's dynamics, both in captivity and in the wild [15].

The determination of female reproductive status can be achieved through the use of techniques that are commonly employed in domestic species; pregnancy is usually determined by means of clinical examination, such as the transrectal palpation of the uterus, ultrasonography, vaginal histology, analysis of serum hormones, and (especially for wild species) through the direct observation of the resulting offspring (post-natal estimates).

All of these techniques have their limitations. Postnatal estimates are sometimes impossible or difficult to obtain, especially for solitary or shy species. Clinical examinations are particularly

difficult and expensive in terms of time and staff when done in the field. Moreover, manipulating the captured subjects causes stress that can consequently produce variations in the animals' normal endocrine profile and present a risk for the animals' health; thus to circumvent these disadvantages, alternative procedures are being studied, including the determination of reproductive status by means of hormonal analysis of feces [16].

In several mammals it has been demonstrated that sex hormones circulating in the peripheral blood are excreted, together with their metabolites, in urine, milk, and feces [17,18]. Feces collection is non-invasive and avoids potential stress-induced variations in hormonal levels. Fecal hormone analysis has already been widely applied in wild species, particularly in ungulates [16-23].

The use of fecal steroid analysis must be subject to controls to ensure that results are analogous to those obtained by other methods, such as haematological testing, which are more complex and difficult to put into effect but that have been validated within the scientific literature. To confirm the suitability of this technique, it is necessary to compare results in the same species for samples taken using fecal testing and at least one additional method (ideally blood testing).

Because the wild boar is the most densely populated ungulate in Piedmont, it is also the most hunted. This is well demonstrated by the statistics of the ‘Regione Piemonte’ which state that about 15,000 animals are shot every year [4]. This species is thus a suitable subject upon which to carry out a quantitative and speedy collection of biological samples for studying a variety of physiological parameters.

The goals of this work were (1) to determine the levels of fecal immunoreactive progestogen (P) and estrogen (E) in female and androgen (A) in male wild boars by radioimmunoassays; (2) to verify the reliability of the results by comparing them with the animals' plasma samples and observed reproductive status; and (3) to determine whether variations exist in the reproductive seasonality of wild boar in two populations living in two different environments within Piedmont with the goal of contributing to the development of correct management strategies to control the wild boar populations.

2. Materials and methods

2.1. Animals and reproductive seasonality

This study was carried out in two areas within the Province of Turin (West Piedmont, Northern Italy); one population was situated in an alpine environment (between 500 and 2000 m above sea level, characterized by hardwood and coniferous forests), and the second was situated in the plain (arable and pasture land and fruit orchards at an altitude of about 300 m above sea level). Wild boars killed during the hunting season (from September through December) and during the demographic control activities exerted by the public administrations (year-round) were considered over a five-year period. A total of 395 animals *(n* = 245 from the plain; *n*= 150 from the mountainous region) were aged either by the extent of tooth eruption and wear [24,25] or by eye-lens weight [26] and the animals' dates of birth were in turn deduced. Animals aged over 10 months were considered adults [22]. The animals were weighed before evisceration and reproductive parameters were recorded (testis weight, number of fetuses, and state of the reproductive tract).

Venous blood samples were collected from 97 adult males (N = 36 from mountainous areas; *n* = *61* from plain areas) in order to determine plasma testosterone levels. For the 61 subjects shot in the plain regions (irrespective of the season), both testes were weighed and the mean of the two weights was tested for correlation with body weight and age. In 64 mature females *(n* = 22 from mountainous areas; *n* = *42* from plain areas) the macroscopic characteristics of the reproductive tract (cyclic ovarian activity, pregnancy, lactation, and immaturity) were determined and venous blood samples were collected to determine plasma progesterone levels.

2.2. Steroid hormone assay for the comparison of fecal and plasmatic concentrations

Blood and fecal samples were collected using standardized techniques from 28 females (17 of which were pregnant) and 26 males, all of which were aged between 4 and 36 months (mean 16.6 ±9.1 months). Fecal samples were collected from rectal ampulla and stored at -20 8C. Immediately following the animals' shooting, blood samples were collected from a large thoracic vessel using a sodium-heparin vacuum tube, centrifuged at 3300 X g for 15 min, and stored frozen as per feces. Subsequently, samples were thawed and prepared for hormonal assays.

Feces were kiln-dried at 80 8C for 24 h in an oven, thoroughly crushed and five parts (0.25 g each) of pulverized feces were put in extraction tubes with a Teflon-sealed cap. 3 mL of diethyl

ether (Carlo Erba, Milan, Italy) were added to each tube and the mixture was vortexed for 10 min. The ether layer was recovered in another tube by decantation after freezing, and evaporated for 24 h. Blood plasma was subdivided in four parts of 1.25 mL each; the aliquots were subjected to extraction by double diethyl ether treatment, with the method described for fecal samples. The fecal and plasmatic extracts obtained were stored at —80 8C.

Extracted samples were brought to 2 mL liquid phase with 97-98% ethanol and PBS (pH 7.4+1% BSA) at a 1:1 ratio. Fecal P, E, and A and plasmatic progesterone (P_4), 17b-estradiol (E_2) and testosterone (T) concentrations were determined by commercial radioimmunoassay kits (DSL 3900, DSL 4800 and DSL 4000—Diagnostic System Laboratories—USA). For each subject samples of fecal and plasmatic materials were used. All analyses were repeated twice.

Inter- and intra-assay coefficients of variation were < 14% for all assays. The P_4, E_2, and T test sensitivities were determined by measuring the least amount of hormone standard consistently distinguishable from the zero concentration of standard and were 0.12ng/mL plasma and 0.96 ng/g feces, 2.2 pg/mL plasma and 17.6 pg/g feces, 0.08 ng/mL plasma and 0.64 ng/g feces, respectively.

The P4 antibody used to quantify fecal P cross-reacts 100% with Progesterone, 6% with 5a-Pregnane-3,20-dione, 2.5% with 11-Deoxycorticosterone and 1.3% with 17a-Hydroprogesterone. The E_2 antibody cross-reacts 100% with Hβ Estradiol and 2.4% with Estrone. The T antibody cross-reacts 100% with T, 5.8% with 5a-Dihydrotestosterone, 4.2% with 11-Oxotestosterone, 2,3% with Androstenedione and 1.9% with Ethisterone.

Serial dilutions (1:4, 1:8, 1:16 and 1:32) of fecal and plasmatic samples from three pregnant, three non-pregnant, and three adult males were assayed to test for parallelism against the standard curve ($P < 0.05$ for all assays). The recovery rates of P_4, E_2, and T added to dried feces were 74%, 69%, and 92% respectively *(n* = 6).

Fecal P and E and plasma P_4 and E_2 concentrations were analyzed in order to subdivide the subjects into pregnant and non-pregnant groups and to verify whether the obtained values were in accordance with the reproductive status of the animals as evaluated by the direct examination of the reproductive organs.

2.3. Statistical analyses

The Kolmogorov-Smirnov test for normality was employed to check whether the data followed a Gaussian distribution. As the normality was not verified, the results were then analysed using the Kruskal-Wallis Test (nonparametric ANOVA) and the Mann-Whitney Test for the comparison of means. In all other cases, ANOVA and Tukey's post-hoc test were used. Spearman's bivariate rank correlation was utilised to correlate fecal and plasma concentrations and Pearson's bivariate correlation was used to examine the relationship between reproductive and individual parameters or hormonal parameters (number of fetuses and fecal progesterone concentrations, testis weight and fecal testosterone concentration or body weight). The Chi-square test was used to compare the birth frequencies. For all statistical analyses, the GraphPadInStat 3.0 software package, (2003) was utilized. Null hypotheses were rejected and results (expressed as mean ± SD) were considered as statistically significant when $P < 0.05$.

3. Results

Fecal P concentrations ranged from 85.6 to 921.2 ng/ g (mean 426.1 ± 210.2 ng/g), whereas plasma P_4 concentrations varied between 0.7 and 38.3 ng/mL (mean 8.9 ± 11.1 ng/mL). The correlation coefficient for fecal and plasmatic concentrations was 0.46 ($P < 0.05$).

Mean fecal P and plasma P_4 (Fig. 1) concentrations found in pregnant females were higher than the mean concentrations found in non-pregnant females ($P < 0.01$).

In 15 pregnant females, fecal P values correlated with the number of fetuses ($r = 0.47$, $P < 0.05$) and ranged from 465.70 ± 80.75 ng/g in triplet pregnancies to 643.16 ± 194.09 ng/g in eight fetuses pregnancies (in two of the 17 pregnant females the plasma samples were lost, and the data on fetuses number were not recorded).

Fecal E levels varied between 40.8 and 830.4 pg/g (mean 212.6 ± 216.2 pg/g), whereas plasma E_2 concentrations ranged from 5.0 to 54.3 pg/mL (mean 10.9 ± 12.2 pg/mL). The correlation coefficient between fecal E and plasma E_2 concentrations was $r=0.58$ ($P<0.01$). The mean value obtained from pregnant females was higher than that from non-pregnant females in both fecal material ($P < 0.05$) and in plasma ($P < 0.01$, Fig. 1).

In the males, fecal A concentrations ranged from 5.0 to 157.6 ng/g (mean 49.5 ± 42.2 ng/g) while plasma T concentrations ranged between 0.7 and 10.8 ng/mL (mean 3.6 ± 3.2 ng/mL). The

correlation between fecal A and plasma T concentrations was r = 0.45 (P < 0.05).

The distribution of births (Fig. 2) shows a seasonal pattern typical of the species in the mountainous area, with 46% of the births concentrated in spring (March-May) and the peak in May (22%). Plain females also gave birth mainly in the spring (33%) with a peak in April (17%); but minor differences throughout the year were present and many births occurred in autumn (Sept-Nov) and winter (Dec-Feb). The seasonal distribution of births results in statistical differences in the spring and autumn (P < 0.05 - Fig. 2). Eighty-three point three percent (15/18) of the pregnant females were found between December and February in the mountainous areas, while 66.7% (6/9) of the pregnant animals in the plain regions were found between September and November.

The profiles of mean T plasma levels of wild boar living in the alpine environment and on the plain are reported in Fig. 3. In the alpine area the maximum individual plasma T levels were observed in autumn when a clear increase in mean concentration occurred (P < 0.01). In contrast, in the plain, plasma T levels in adult males were high and relatively constant throughout the year, averaging ≈7 ng/mL, with a slight (but not significant) reduction occurring during the summer months (4.8 ng/mL).

Plasma T levels were lower in the mountain boars compared to those living on the plain. During the spring, the levels in mountain and plain dwelling animals were respectively 1.7 and 7.4 ng/mL (P < 0.05), in the summer 1.5 and 4.8 ng/mL (NS), and in the winter 1.5 and 6.9 ng/mL (P < 0.05). In the autumn, however, both populations showed the same mean concentrations (7.4 ng/mL).

For the 61 boars from the plain areas the correlation coefficient between testis weight and plasma T concentrations is r = 0.60 (P < 0.01) whereas the correlation between testis weight and body weight is r=0.84 (P<0.01). T concentrations changed in relation to age (r = 0.50, P < 0.01 as well as testis weight (r= 0.89, P < 0.01; Table 1).

In boars older than 30 months, no correlations existed between testis weight and plasma T concentrations or between testis weight and body weight.

4. Discussion

Our results show that changes in the fecal steroid concentrations are similar and parallel to those in the plasma steroids; the correlations are statistically significant. It is known that one of the most important difficulties when working with fecal steroids is to be sure that changes in the fecal hormone concentration actually reflect variation in blood concentration, and are therefore physiologically relevant. Our results confirm that this is indeed the case. As observed in other mammalian species [27-29], steroid concentrations in feces are several orders of magnitude greater than that of the parent hormone in the blood. However, serum values can be predicted from fecal data.

Fecal P and plasma P_4 concentrations in females confirmed pregnant by the presence of fetuses within the uterus were never lower than 170 ng/g and 2 ng/mL, respectively. Fecal P concentrations were higher than 380 ng/g in 88.2% (15/17) of these females, whereas this occurred in only 11.1% (1/9) of the non-pregnant females. This fecal P value can therefore be considered as useful for distinguishing the pregnancy status in females.

In the 15 pregnant females examined, progesterone levels directly correlated with the number of fetuses. Such a correlation has also been reported in sheep (during early pregnancy), goat, and pig [27,30,31], presumably due to the increased number of corpora lutea as the fetal placental unit is not a major source of progesterone in this species [32-35].

Fecal E values were higher than 100 pg/g in 82.3% (14/17) of the pregnant sows. In non-pregnant animals, the data appear to be more homogeneous and are clearly lower, but 12.5% (1/8) of the sows had values higher than 100 pg/g. For this reason, fecal E concentrations *per se* do not seem particularly useful for detecting pregnancy status.

However, high fecal concentrations of P and E (greater than 170 ng/g and 100 pg/g respectively) were observed in 70.6% (12/17) of pregnant sows and were never observed in any of the non-pregnant sows and could therefore be considered as a strong indication of a pregnant status.

In males, the lowest T values were observed in young subjects (< 1 yr old) and the highest values were found in adult males (> 30 mo). Low concentrations were also observed in some adult males (only those < 30 mo), confirming that the levels of testosterone in adult animals can also be influenced by many factors, such as the presence of females in their breeding season, body condition, and environmental factors (i.e., photoperiodism, temperature, food availability, and social factors) [36-38].

Mean body and testis weights are highly correlated and increase with subject age, as is known to occur in response to normal body growth [1,5,39].

The correlation between testis weight and hormone levels is less pronounced, likely due to differences in steroidogenic activity linked to seasonal rhythms or reproductive status [37]. The data from the calculated distribution of births and from the autopsies and plasma assays of culled females converge to suggest the existence of an influence of climatic conditions on reproductive seasonality. Compared to the plain, in the alpine area a higher concentration of births in the spring may represent an adaptive mechanism for young survival. The differences in the reproductive period recorded in the plain probably mainly reflects different environmental conditions, but it may also be caused by the introduction of some adult animals into the wild from breeding farms; boars bred in captivity are known to display reproductive cycles analogous to those of domestic pigs. In fact, crossbred animals have lost the seasonality of reproduction and are highly prolific, with consequent increase in deliveries and new-borns during the year [1].

The lower rate of summer births in both populations is probably a consequence of the high number of births in the spring.

The profiles of mean T plasma levels of wild boar living in the alpine environment and in the plain are different. Our data suggest that the autumnal raise in T concentrations in mountain dwelling males begins before the fertile mating period that occurs during the winter months as deduced from birth dates. Indeed, testosterone levels are known to be at their highest during the pre-mating and mating season that fall in autumn and early winter according to the photoperiodic influences [40—42]. Similar patterns have also been observed in other seasonal species such as Bighorn sheep *(Ovis canadensis)* [43], Nubian ibex *(Capra nubiana)* [44], Alpine ibex *(Capra ibex)* [45], and goat *(Capra hircus)* [46].

Furthermore, the lack of a clear seasonal pattern in the mean T plasma levels in the plain males supports the data from the distribution of births, while in the mountain the presence of births referable to periods in which the testosterone levels are low may be linked to altitudinal displacement made by the pregnant females to reach optimal areas for feeding and shelter (unpublished results). These observations strengthen the hypothesis that somewhat different patterns in reproductive rhythms exist between the two geographically distinct populations of wild boars.

5. Conclusions

Measuring sex steroids in feces offers the possibility of monitoring reproductive status in wild animal species through the use of a simple sampling technique. Fecal samples may be easily collected and preserved without requiring repeated and stressful capturing, handling, and/or blood collection procedures. We propose this non-invasive method for the assessment of pregnancy in females and male sexual activity in wild boar. The results support the applicability of this method in wild boar in order to obtain new biological knowledge and the development of best-practice management strategies for such populations. This becomes especially important in light of the problems arising from the demographic expansion of this species in areas of human activity (e.g., agriculture and car accidents involving wild boar on the roads), such as those occurring in Piedmont and other Italian regions.

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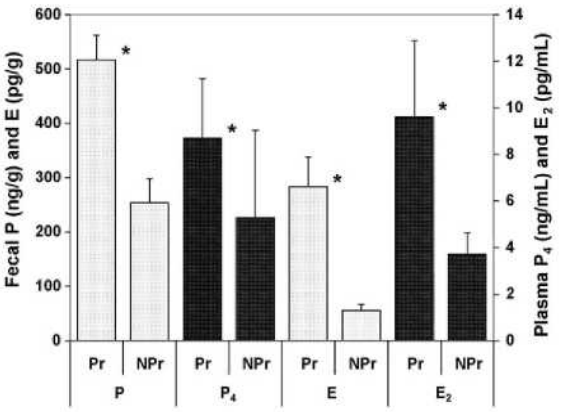

Fig. 1. Fecal and plasma sex steroids - A comparison between pregnant (Pr) and non-pregnant (NPr) females. E = Estrogens, P = Progestogen, E_2 = Estradiol-Hβ, P_4 = Progesterone. Asterisk indicates $P < 0.05$ between pregnant and non-pregnant groups.

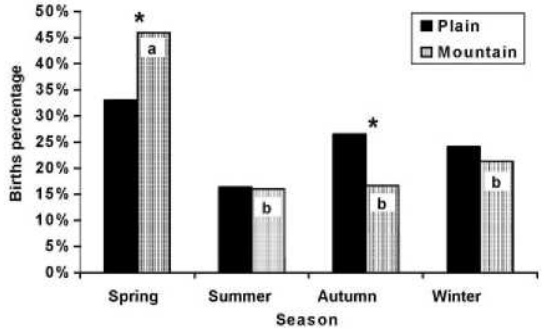

Fig. 2. The distribution of births through the seasons in two populations of wild boars living in different environments. Different letters indicate $P < 0.05$ between seasons in the group, asterisk indicates $P < 0.05$ between the groups in the season.

Fig. 3. Plasma androgen levels (mean ± SEM) of wild boars living in an alpine environment (dashed line) or in plain (unbroken line). Different letters indicate $P < 0.05$ between seasons in the group, asterisk indicates $P < 0.05$ between the groups in the season.

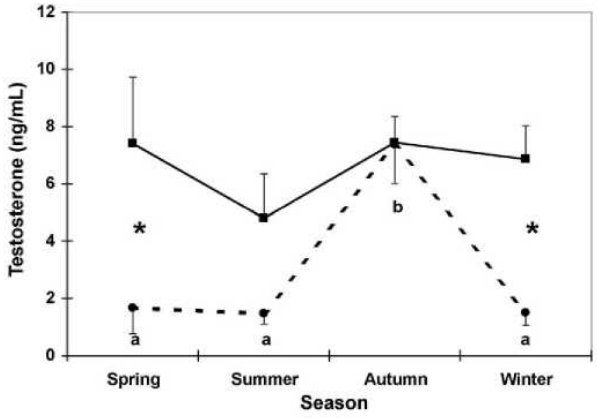

Table 1
Mean values of plasma Testosterone (T), testis and body weight in different age groups of wild boars.

N. subjects	Months of age	T (ng/mL)	Testis weight (g)	Body weight (kg)
6	4-6	4.78	27.86	12.44
13	7-12	3.57	55.66	24.88
15	13-18	6.76	103.09	31.24
16	19-30	9.76	175.31	51.55
11	>30	10.50	210.39	52.63