

A non-invasive, improved RIA and overt observation in the study of singleton Apennines' wolf (*Canis lupus*) reproductive behavior

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Abstract. The analysis of fecal hormones allows a close but non-invasive monitoring of animals avoiding the stress of restraint/capture, which in turn can upset animals' hormonal profile. Steroid hormone progesterone was analysed in three singleton, female grey wolves of different age, belonging to the endangered species of the Apennines' *Canis lupus*. The analysis was carried out during the breeding season by using an improved radioimmunoassay on samples collected on the field. To reduce the stress to animals and danger to people, the overt observations were carried out by operators who were already familiar with the animals, saving the money of a camera-monitoring-system. Concurrently, a male and a female gray wolves housed together were monitored as a control. The results indicated the importance of dehydration of fecal samples before the extraction with petroleum ether, which was shown to be more efficient than diethyl ether, and that pre-treatment with methanol greatly enhances extraction ($p < 0.01$). Females of Apennines' grey wolf showed the first sign of oestrus by a vaginal blood loss, that was easily detected on the ground; the analysis carried out on fecal samples revealed a rapidly declining luteal phase, with P4 metabolites reaching the basal values of a non-cyclic female. In the matter of welfare, behavioural observations on Apennines' grey wolf showed that unpaired animals, although familiar with the operators, failed to display a sexual social behavior during the reproductive season, that is the behavioural signs were hidden in overt observational situation.

Key Words: animal welfare, *Canis lupus*, fecal steroid, progesterone, reproduction.

Riassunto. Le indagini ormonali condotte su campioni fecali permettono un monitoraggio accurato degli animali senza che questi vengano sottoposti a stimoli stressanti quali contenimento e/o cattura i quali, di norma, sono responsabili di modificazioni del profilo endocrino. Tre lupi grigi femmina in isolamento appartenenti al Lupo Appenninico (Apennines' *Canis lupus*), un sottogruppo autoctono italiano minacciato di estinzione, sono stati monitorati durante la stagione riproduttiva attraverso la ricerca di metaboliti fecali del progesterone, insieme all'osservazione contemporanea del comportamento di una femmina alloggiata insieme ad un maschio. I risultati incoraggiano ad usare l'essiccamento dei campioni fecali prima dell'estrazione dei metaboliti steroidei con etere di petrolio; quest'ultimo ha mostrato capacità di estrazione migliore rispetto al dietil etere, che può essere ulteriormente migliorata attraverso il pretrattamento con etanolo ($p < 0.01$). Le femmine di lupo grigio appenninico hanno mostrato i segni dell'estro attraverso una perdita ematica abbastanza copiosa da essere rilevata al suolo; le femmine alloggiate in condizioni di isolamento hanno presentato valori di progesterone rapidamente degradanti verso i livelli basali di animali non ciclici. Nonostante tutti i soggetti fossero usi al contatto con gli operatori, le osservazioni etologiche, importanti per la valutazione del benessere animale, hanno permesso di verificare la mancata esibizione di comportamenti estrali nelle femmine mantenute in isolamento rispetto alla femmina alloggiata in presenza del maschio.

Parole Chiave: benessere animale, *Canis lupus*, steroidi fecali, progesterone, riproduzione.

Introduction. The conservation of biodiversity is one of the major issues of the 21st century, being yearly more and more increasing the rate of animals extinction; unfortunately, this escalating loss is a serious threat to human being and to the future of the planet Earth itself. For instance, among the species endangered of extinction, the Italian grey wolf (*Canis lupus*) can only survive thanks the protection of wild life conservation organisation. IUNC (International Union for Conservation of Nature) classification is still fixed on the "least concern" category, but the Wolf Specialist Group admits that the wolf "has become extinct in much of Western Europe, in Mexico and much of the USA", and in Italy the wolf population is threatened by human persecution (Mech & Boitani 2004, 2008). Italian law protect grey wolf's population by the President Decree n. 357/1997 and at transnational level the wolf is included in the Appendix II of the CITES (Washington Convention on animals and plants threatened species).

The Apennines' grey wolf (Apennines' *C. lupus*), although closely related to his European relatives, has suffered from a long genetic isolation from them; probably for this reason, the present-day population of all the Italian wolf genotypes has been assigned to a single group, supporting their genetic distinction (Lucchini et al 2004). In Italy, the numerically reduced population of Apennines' grey wolf can hardly be protected since there are some risk factors, including inbreeding depression and genetic variability loss (Ciucci & Boitani 1996). Every subject is therefore precious to ensure the survival of the species. Unfortunately, to protect rescued wolves (for instance from accidents, poisoning or, more frequently, human hunting) it is often necessary to house them in captivity for long periods; sometimes, when they are unable to return in the wild, the housing is lifelong. So far, the Italian grey wolves have been poorly studied except for habitat, home range activity, and dietary behavior (Boscagli 1985; Boitani & Ciucci 1995; Ciucci & Boitani 1997; Gilio et al 2004; Marucco et al 2008), and less for their reproductive physiology, a difficult matter to study in the field. On the other hand, even though captivity makes any experimental design easier, it brings ethical, safety and welfare concerns, that are well-grounded for endangered animals maintained in unnatural conditions. For example, dealing with wolves coming from the wild, i.e. social animals, it is hazardous to join together different subjects without exposing them to hierarchic aggression; on the contrary, leaving them in singleton could lead to stress and suffering. In this uncertainty, the rescue centres often tend to isolate animals to accelerate their recovery and make their management easier. In these conditions, however, most of the animals are subjected to a reduction of reproductive performance (Maia & Gouveia 2002; Mallonée & Joslin 2004) and only a continuous and tight surveillance can make it possible to assess the reproductive status of the animals. In this context, the monitoring of wolves reproductive health becomes of pivotal importance.

Since in most cases wildlife conservation is performed by non-profit organisations, the scarcity of economic resources imposes that any routinely used methodology must be: i) effective and reliable, ii) easy to be performed and, more importantly iii) not expensive. Moreover, whatever procedures are used, they must avoid to induce the insurgence of stress in these animals, that are, and that must remain, wild animals.

Differently from domestic species, in fact, it is extremely difficult, as well as ethically unacceptable, to train wild wolves for those practices, for example injections, because of their shy nature. Therefore, when physiological measurements have to be done, such as daily assessment of hormone levels, non-invasive techniques should be undertaken, that do not require animals' capture.

The reproductive behavior of the Apennines' grey wolf has been described in Italy by Boscagli (1985): wolves are seasonally monoestrous, with a unique oestrus per year, and mating season is strictly related to climatic and environmental factors, including latitude, in Italy from February to April. Puberty in female occurs at nearly twenty-two month old, becoming apparent through vaginal swelling and blood discharge, visible as reddish drops on the soil, fallen leaves and other substrata. As reported by Boscagli (1985), the reproductive season in this Apennines' *C. lupus* is characterized by increasing nervousness, ritualized or true fight, male-female courtship and approaches also between subordinates, although (almost always) only the alpha couple mates and cubs once a

year. The proestrus lasts approximately from ten to twenty days; oestrus goes on for one week and ovulation occurs spontaneously, but the precise timing is not known.

Starting from these bases, the aim of this study was to propose and to validate a protocol based on non-invasive techniques, such as the observation of animals in their enclosure environment and the measurement of hormonal levels in the feces samples. In particular, the assessment of progesterone was performed because this hormone is produced by preovulatory follicle and by corpus luteum (CL); as a result, it is a reliable marker of ovarian activity.

The isolation of gonadal steroids metabolite from feces allows to check the phases of reproductive physiology (Gudermuth et al 1998; Velloso et al 1998; Goodrowe et al 2000; Brown et al 2001; Valdespino et al 2002; Cerda-Molina et al 2006; Songsasen et al 2006) and to assess the relationships between behavior and endocrine factors. Feces of most vertebrates contain metabolites of major steroids (Schwarzenberger et al 1996; Keay et al 2006) and in several species a close correlation between steroids levels in blood and different secretions has been established (Shideler et al 1993; Berkeley et al 1997; Hay et al 2000; Valdespino et al 2002). In our study, to cut the costs of analytical procedures, it was tested if the assessment of just progesterone could be effective for the diagnosis of reproductive activity. In addition, two different extraction methods were compared, in order to determine the most effective technique.

Material and Method

Animals. The study was carried out on three singleton and one paired females of Apennines' grey wolves, maintained at the National Wildlife Refuge of Popoli in central Italy. The three singleton animals were chosen because two of them were certainly cyclic (consequently, they were assumed to be the positive control) and the third female was surely acyclic (negative control). More in particular, the wolf 1 was nearly ten-month-old at the time of sampling, and she was monitored because at that time it has been demonstrated that Apennines' grey wolf females are non-cyclic (Boscagli 1985); female 2 was nine-year-old, rescued together with some young wolves in Tuscany and then transferred to Popoli. Female 3 was three-year-old at the time of this study and was recovered from a car accident. The two paired wolves (female 4 and male 1) were both mature and of proved fertility; they were considered as a control for the exhibition of sexual behavior but not for fecal hormone extraction and detection, being quite impossible to discriminate between female and male feces.

In the National Wildlife Refuge of Popoli, the wolves are housed outdoor in singleton, in 0.25 Km² wire netting enclosures (the couple in a double spaced enclosure), enriched with puddles for bath, and provided with access to den boxes and wood resting-place; the animals are feed daily with lamb meat, vitamins-supplemented, and always available water.

In the present study, the enclosures inspection was carried out twice a day, starting every morning before the collection of the samples, then in the late morning or before the sunset for ten to fifteen minutes. The signals of vaginal discharge were checked both on females, when visible, or on the ground when the animals hid themselves inside the enclosure. The enclosures were faraway enough to prevent the wolves to come up to each other; therefore, no contact was possible apart, obviously, that concerning visual, vocal and olfactory senses.

Samples collection, fecal extraction and analysis. Fecal samples (n = 235) were collected every day (if present) in the singleton wolves enclosures only, during the breeding season, that is February-April at the latitude of the central Italy (Boscagli 1985). Fecal samples instead were not collected in the paired wolves enclosure but, for the female 4, only the behavioural sign of oestrus were recorded. During the observational period, the first day of blood discharge was recorded as day zero. Only fresh feces were considered: an amount of approximately 30 g from each sample of fecal mass was frozen immediately at the laboratory of the National Wildlife Refuge, and transferred weekly to the lab to be stocked at -80°C until analysed. The problem of different lipid distribution in wet material that could have lead to artifactual values during

the steroid analysis was avoided by desiccating the fecal samples, although the technique was time-consuming (Brown et al 1994). In brief, after thawing at room temperature, each sample was checked for integrity; unsuitable or openly musty samples were discarded. Then, 10 out of 30 g of feces were taken and powdered in a petri dish with 30 mL of PBS (Phosphate-Buffered Saline: NaCl 150.1 mmol/L, KH₂PO₄ 1.998 mmol/L, Na₂HPO₄ 8.002 mmol/L). The diluted sample was filtered to separate the most rough materials and two aliquots of 6 mL each were put in new petri dishes to be dehydrate in oven at 60°C for 24 h, and finally stocked at -20°C. All reagents used, otherwise specified, were from Sigma-Aldrich, Dorset, UK.

In order to optimize steroid extraction and detection, we tested two different solvents such as diethyl ether (DE; Hoffmann & Möstl 2001; Ishikawa et al 2003) and petrol ether (PE; Hay et al 2000), both alone and with 20% methanol pre-treatment (Shideler et al 1993). In brief, after the addition of 4mL of one of the two chemicals, samples were properly mixed by agitating them on a rotating dish (Maxi Mixer 714, ASAL, Milano, Italy) for 2h, then frozen at -80°C and finally decanted: after ether evaporation, dry material was re-suspended in ethanol (Normapur, VWR International, France) and stocked at -20°C until assay. A validated radioimmunoassay was used for the measurement of progesterone (Tamanini et al 1985): 50 uL of the diluted extract were dried, dissolved in 100 uL RIA-phosphate buffered (Na₂HPO₄ 74.26mmol/L, EDTA-Na 12.49 mmol/L, NaN₃ 7.69 mmol/L) containing 0.1% bovine serum albumin, pH 7.5 and shaken for 5 min. After adding 1,2,6,7-³H progesterone (Amersham Biosciences, UK) at a rate of 48 pg/tube, and rabbit antiserum raised against P₄ at the working dilution of 1:4,000, samples were incubated overnight at 4°C. Anti-progesterone antibodies Rb-128 were kindly supplied by the Department of Morphophysiology and Animal Production (University of Bologna, Italy); the rabbit antiserum specificity (in %) was: Progesterone 100%, 5a pregnan 3-20 dione 64,4 %, 11aOH Progesterone 9.7%, 17aOH Progesterone 1.5%, 20aOH Progesterone 0.3%, Cortisol 0.05%, Testosterone < 0.002%, 17-b estradiol < 0.0001%. The day after, 1mL charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer) was added to the tubes; after 15 min at 4°C, the tubes were centrifuged for 15 min at 3,000g., the supernatant decanted and radioactivity was measured immediately using a beta scintillation counter (LS 6000IC; Beckman Instruments, USA). Validation parameters of the analysis were: sensitivity 49.3%, intra and inter-assay variability were both < 10%.

Extraction efficiency of the two different solvent was measured by a recovery test (five replicates), carried out by adding 125, 250, 500 or 1000 pg of ³H-progesterone to 100 mg of dehydrated feces in 2 mL of PBS plus 4 mL of PE or DE, both alone and with methanol pre-treatment; after mixing carefully on a rotating dish for 20 min and frozen at -20°C, the solvent radioactivity has been counted at the beta counter together with 4 mL of scintillation fluid.

Data analysis. Data were expressed as a means ± s.d. of three independent experiments, each of them carried out in duplicate. The conformity of data to the Gaussian distribution was tested by Shapiro-Wilks W test and the differences between different samples have been evaluated using the analysis of variance (ANOVA for repeated measures) and values of P < 0.05 and < 0.01 were considered significant and highly significant, respectively. Standard curves were obtained with a logarithmic fitting to the experimentally-determined values and the coefficient r² was determined. For both the evaluations, Statistica 6.0 software was used.

Results and Discussion

Wolves observation. During the study, we registered the temperature values, which ranged from -2/0 °C to 9/19°C and the day length, which increased from 11.25 h to 14.00 h (sunrise from 6.45 to 5.23 and sunset from 18.10 to 19.20) from February to May. In this period, the operators did not notice any sign of social reproductive behavior in singleton female wolves during the enclosures inspection. Very often the wolves remained motionless and faraway from the routes usually scoured by humans, confirming their diffident wild nature. In the meantime, the operators observed the soil searching for

blood drops or fresh feces, and recording the first day of recovery as "day zero" of oestrus; this date occurred on March 9th for wolf 3, and on March 16th for wolf 2. No traces of blood were observed during the experimental period in the wolf 1 enclosure. The paired female 4 showed instead some signs of nervousness and a repeated licking of genitals, performed indifferent to human presence; the day zero for this wolf was recorded on March 8th, but the courtship of the male toward her was obvious since ten days before, that is at the end of February.

Solvent test. In this study, the P₄ extraction efficiency from feces was $32 \pm 3.1\%$ for DE and $39.8 \pm 2.7\%$ for PE, the latter being more efficient ($P < 0.05$) and thus it was chosen for all the subsequent analysis. Moreover, pre-treatment with methanol significantly improved PE extraction to $48.7 \pm 3.1\%$, vs 33.5 ± 2.5 for DE ($P < 0.01$). Each sample of dehydrated feces was analysed in triplicate and the values obtained were corrected both for the diluting factor and extraction efficiency, as suggested by Weingrill et al (2004).

Fecal P₄ detection. The individual rhythm of progesterone metabolites excretion for each wolf has been reported in Figs 1-3, where the bars indicate the first day of vaginal blood loss. The wolf 1, did not show any sign of oestrus during the whole experimental period but, in spite of this evidence, the samples recovery was continued until the end of observation (starting from February 7th to May 22nd). The P₄ values in the harvested feces (94 samples, most of them suitable for analysis) ranged between 109.7 ± 11.2 to 538.3 ± 43 ng/g, indicating a non-cyclic, prepubertal animal (see Figure 1). Wolf 2 was monitored from March 2nd until May 3rd. The excretion rhythm in wolf 2 showed a concentration ranging between 62.05 ± 2.68 up to 320.71 ± 34.84 ng/g before day zero. On March 15th however, one day before the beginning of vaginal discharge, P₄ levels in feces raised to 1556.98 ± 389.59 ng/g (see Figure 2).

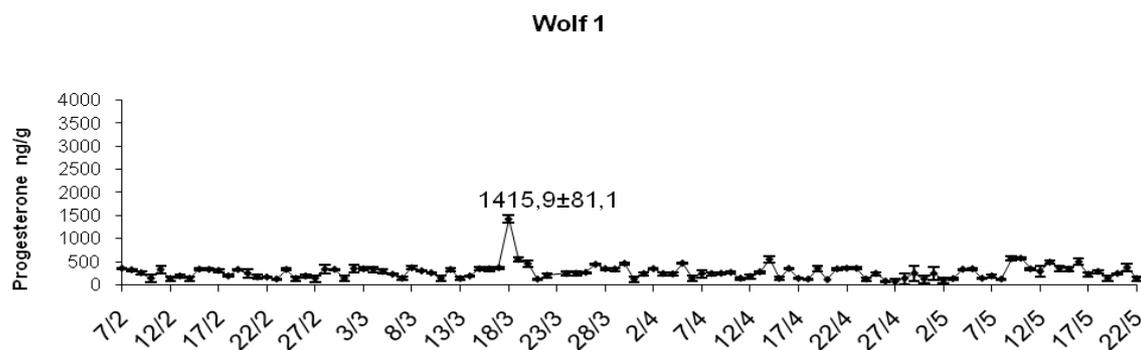


Figure 1. Longitudinal profile of P₄ excretion in wolf 1 monitored from February 7th to May 22th. Values are expressed as mean of three reading \pm s.e.

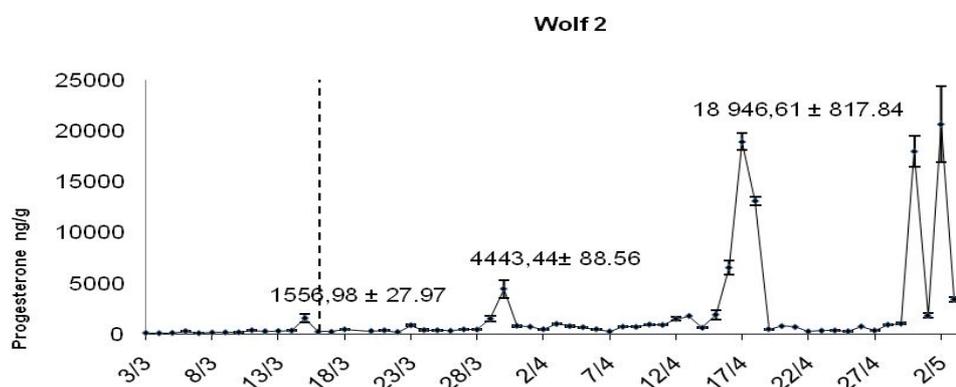


Figure 2. Longitudinal profile of P₄ excretion in wolf 2 monitored from march 3th to may 3th. Values are expressed as mean of three reading \pm s.e. Vertical bar indicates the day of first vaginal discharge (zero day).

Then the P₄ level points out a net increase in two weeks of time, followed by a decrease, to raise and drop again until the end of sampling. Wolf 3, monitored from February 24th to May 3rd, did show the first sign of vaginal blood discharge on March 9th. The pick level of P₄ detected in this animal was 42 802.4 ± 1747.4, and occurred on March 20th, eleven days after the detection of proestrus (see Figure 3).

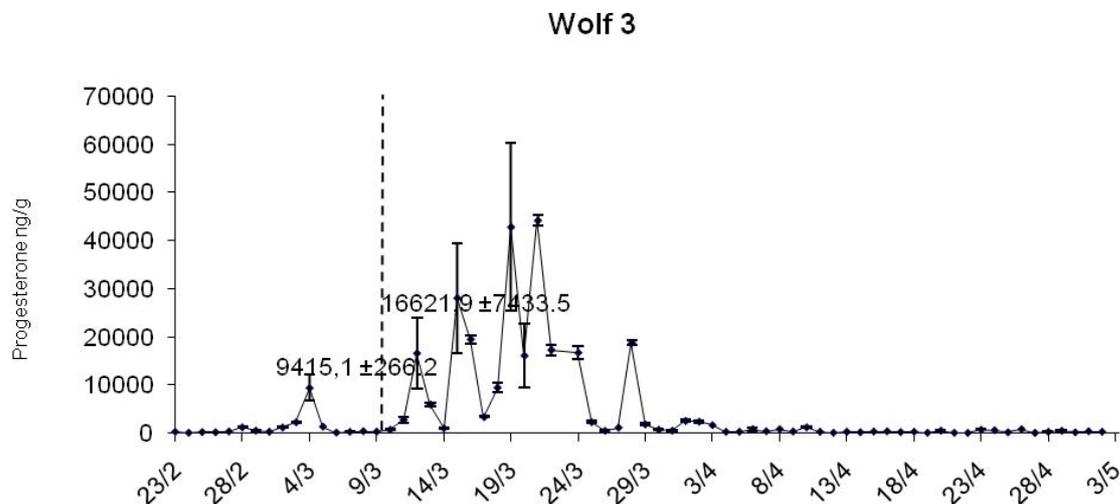


Figure 3. Longitudinal profile of P₄ excretion in wolf 3 monitored from February 24th to May 3th. Values are expressed as mean of three reading ± s.e. Vertical bar indicates the day of first vaginal discharge (zero day).

Discussion. Our century is characterized by a more and more increasing danger of biodiversity loss. Two different aspects are conflicting: the urgency to rescue the species in danger of extinction and the inexorably limited economic resources. In this context we considered the hypothesis of using a novel protocol to detect the reproductive performance in captive wolves, that could have been impaired by captivity conditions, as stated by other authors (Maia & Gouveia 2002; Mallonée & Joslin 2004). In the meantime, we also try to detect if the condition of strict captivity could have detrimental effects on wolf sexual behavior.

The protocol we proposed follows two main criteria: a higher cost/benefit ratio, and the coupling of two different methodologies: i) the analysis of a hormone involved in the reproductive activity and ii) a stressless behavioral indicator obtained by operators that captive animals were used to see; by putting together such two methodologies, we try to increase the predictivity power without adding the costs of recording, transcribing, or storing data. In addition, the most effective extraction method for P₄ was tested.

The results showed that the extraction method with PE assured the best result, making possible the recovery of more than 40% of the hormone metabolites, a quite satisfactory finding. Data from different protocols demonstrated values ranging from 17.1 ± 1.6% (Cerda-Molina et al 2006) to 80 ± 10% (Songsasen et al 2006), which confirm the acceptability of our result. It is difficult to speculate about the reason why the extraction protocol including PE was more successful than that of DE: in fact, PE is a group of various volatile, highly flammable, liquid hydrocarbon mixtures of alkanes, e.g. pentane, hexane, and heptane, whereas benzene is a cyclic, aromatic hydrocarbon, C₆H₆. As a result, being PE a mixture of different molecules with different polarity characteristics, it is impossible to ascribe the chemical proprieties of the compound to one component rather than another.

In our study, the levels of P₄ in feces are markedly higher than those reported by other authors (Walker et al 2002; Songsasen et al 2006) tanks to the extraction method, the higher reactivity of our antibody with the predominant P₄ metabolites in wolf feces, and the sensibility of the experimental methods. In particular the assay we used, the radio immuno assay, can be considered more sensitive than other methods. After all, the major problem affecting different fecal steroid quantification is the breakdown of steroids

by gastrointestinal bacteria and exogenous microbes (Beenher & Whitten 2004), and about that, it is known that the concentration of molecules detected by different assays are the consequence of i) the enteric flora metabolic activity, that markedly differs among different animals, caged in different conditions and fed on different diet (and in the same animal in different moments) and ii) environmental factors, such as external, biological and climate agents. All of these conditions are then highly variable among different studies and inside the same experimental set-up; our study, however, by a daily recovery of the fecal samples and a standardized dietary condition, minimized a few quantification differences. Once established how to assess the progesterone levels, it was found that the observational and hormonal data were in perfect agreement.

The evidence of a pre-ovulatory rise in fecal P₄ metabolites in Songsasen and his colleagues work is strictly correspondent with our findings, and are likely signals of a precocious follicular luteinisation in maned wolf (Songsasen et al 2006) as in the bitch (Concannon et al 1977). Indeed, as reported by Seal et al (1979), the female wolf experiences an increase in serum P₄ levels some days before the preovulatory surge of gonadotropins, thus on the basis of the demonstration of the close correlation between the level of steroid in blood and feces, we can argue that also Wolf 2 and 3 experienced a similar condition. Seal et al (1979) explained this preovulatory surge of P₄ by admitting they could have influenced animals hormonal profile because their data were achieved from strictly captive animals after pharmacological restraint and anaesthesia carried out once every 2-3 days. The fact that in our study it was possible to determine a similar endocrine pattern in more physiological conditions could improve captive animal welfare when we need to perform a hormonal analysis.

As regards reproductive social behavior, we were not able to detect any sign in singleton Apennines' females. We cannot argue if the same wolves we considered in our study could have express sexual behavior in the wild, nevertheless, we just could assume that our captive wolves did not behave as wolves observed in the wild during the breeding season (Boscagli 1985), nor as the only paired female housed in the same centre. This could have lead to consider those animals as non-cyclic, based on a superficial observation. Hormonal changes experienced during the oestrus normally elicit a typical increase in sexual behavior, and we were able to record it in the female wolf 4 and her male, that were housed in the neighbour enclosure. We cannot assume, however, if captive lonely wolves did not experience the typical repertoire of oestrus because of the captivity or because they were not paired.

Our findings reflect the real reproductive settlement of the examined wolves, in other words the positive controls and the negative ones were confirmed by the analysis we carried out. In conclusion the protocol we proposed allows the determination of sexual activity in wolves combining two different kind of examinations: the observation of animals and the progesterone quantification in fecal samples. This protocol, moreover, is easily reliable and economical and it could be regarded as an additional piece in the bigger puzzle of the management of rescued wolves, as a practical approach to captive animals welfare.

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