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SCUOLA DI DOTTORATO IN SANITÀ E PRODUZIONI ANIMALI: SCIENZA, TECNOLOGIA E BIOTECNOLOGIE

DOTTORATO DI RICERCA IN PRODUZIONI ANIMALI
Ciclo XXIV

WILD RED DEER (*Cervus elaphus*, Linnæus, 1758) POPULATIONS STATUS ASSESSMENT: NOVEL METHODS USING HAIR

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Ad Andrea ed Emma

"Il fatto che l'attività svolta in modo così imperfetto sia stata e sia tuttora per me fonte inesauribile di gioia, mi fa ritenere che l'imperfezione nell'eseguire il compito che ci siamo prefissi o ci è stato assegnato, sia più consona alla natura umana così imperfetta che non la perfezione. Considerando in retrospettiva il mio lungo percorso, quello di coetanei e colleghi e delle giovani reclute che si sono affiancate a noi, credo di poter affermare che nella ricerca scientifica né il grado di intelligenza né la capacità di eseguire e portare a termine con esattezza il compito intrapreso siano i fattori essenziali per la riuscita e la soddisfazione personale. Nell'una e nell'altra contano maggiormente la totale dedizione e il chiudere gli occhi davanti alle difficoltà: in tal modo possiamo affrontare problemi che altri, più critici e più acuti, non affronterebbero."

Rita Levi Montalcini (1909-2012)

Contents

Abstract

The assessment of free-ranging wild red deer (*Cervus elaphus*) populations status is an important tool in wildlife management, because of, in some areas, this species reaches high densities, which can increase the occurrence of road accidents, of damages to agriculture and forest regeneration, and, not last, can reduce the fitness of the species itself. In fact, high density populations compared to low density ones usually show lower levels of fertility, higher prevalence of disease, higher mortality, worst general body conditions and nutritional status. Nevertheless, collecting samples to assess free-ranging populations status is often difficult. Hair is a safe, readily available, and easy to store and transport matrix, and hair sampling does not involve pain or infection risk for the animals. Furthermore, hair assay provides a long-term endocrine profile. Thus, this matrix could be useful to assess long term cortisol accumulation and other hormonal substrates of social trends. Furthermore, hair can be an important indicator of accumulation of environmental pollutants in ecological, clinical and hygienic studies. In this thesis three studies were carried out, concerning respectively the extraction of cortisol, progesterone (P4) and arsenic from hair. We show how the analysis of hormones or other substances in hair constitutes a highly promising and reliable method for assessment of substances secretion over extended periods of time in free-ranging red deer. In particular, our findings suggest that i) hair cortisol concentration provides a good index of long-term HPA axis activity and allostatic load; ii) hair progesterone concentration, in combination with other sexual hormones concentrations in hair and biometric measures, may contribute in the future to develop a reliable and easy pregnancy test for free-ranging red deer; iii) hair arsenic concentration could be analysed, not only in order to assess wild populations status, but also

to control wild animals contamination, in biomonitoring investigations or in health programs. In conclusion, the assessment of hormones and micro-elements in the hair seems to be an interesting tool for future wild species management.

Keywords

Red Deer, *Cervus elaphus*, Hair, Cortisol, Progesterone, Arsenic.

1 Introduction

1.1 Objectives

The objectives of this thesis are:

i) To review briefly the literature about stress and welfare, nutritional status, body condition, and reproduction in animals in general and, in particular, in wild red deer;
ii) To investigate the possible use of hair to assess the status of wild populations;
iii) To discuss the future applications and perspectives of this method in wildlife management.

1.2 Thesis structure

In Chapter 2 we propose an introductive literature review, in particular referred to wild red deer (*Cervus elaphus*), concerning i) stress, allostatic load and welfare; ii) nutritional status and body condition; iii) reproduction; and iv) biomonitoring.

In Chapter 0 we provide i) an introductive part, regarding the use of hair as matrix for the extraction of hormones and other substances and the description of the study area; ii) three experimental studies concerning respectively cortisol, progesterone and arsenic assessment in hair.

In Chapter 4 we summarize the conclusion and put forward proposals for future possible investigations starting from our findings.

2 Literature review

2.1 Stress and Welfare

2.1.1 Stress

The term "stress" is commonly used to indicate a negative and undesirable condition of an individual; actually, some stresses are necessary and beneficial to the animal. Furthermore, stress cannot be totally avoided in a complex real world (Squires, 2003). The mechanism of stress may have evolved for coping with threats to the survival and well-being, to maintain animal homeostasis [1] (i.e. allostasis)[2].

Considering its importance, it is difficult to give an univocal definition of this concept: stress has no defined aetiology or prognosis.

A modern interpretation defines stress as "the biological response elicited when an individual perceives a threat to its homeostasis" (Moberg, 2000). The threat is also called "stressor" and can include a number of psychological, environmental, or physiological stimuli (Peterson *et al*., 1991).

Stress can be more or less harmful, depending on the capacity of the organism to cope with the negative situation and to re-establish

[1] Homeostasis is the stability of physiological systems that maintain life (e.g. pH, body temperature, glucose levels) (McEwen and Wingfield, 2003).
[2] Allostasis is achieving stability through change. It is a system that maintain the systems that are essential for life (i.e. homeostasis), by mediators such as, but not confined to, hormones of the hypothalamo-pituitary-adrenal axis, catecholamines, and cytokines (McEwen and Wingfield, 2003).

homeostasis. “Distress” (or “bad stress”) is “a biological state where the stress response has a deleterious effect on the individual’s welfare” and it is different from a non-threatening stress response (also called “eustress” or “good stress”) (Moberg, 2000).

The problem is to determine when stress becomes distress and how to measure it in animals.

Along the time, a number of indicators and measures of stress have been used (see paragraph 2.1.7), but none of them is fully satisfactory, and the reason is that it is impossible that a single indicator will be appropriate for all types of stressors (Moberg, 2000). Furthermore, biological responses to threatening and innocuous stimuli can be similar, so care must be taken in interpretation of the results.

The response to a stressor requires the animal to expend energy (biological cost of stress), and this can be very important in wild animals, living in poor environmental conditions, or during particular life stages (e.g. reproductive period, pregnancy, lactation) (Reeder and Kramer, 2005). When the biological cost of stress diverts resources away from physiological functions (immune competence, reproduction, growth), the animal has experience of distress (Moberg, 2000).

2.1.2 The mechanism of stress

The first studies about the mechanism of stress were conducted from Cannon and Selye, in the first half of the past century. They proposed two different kinds of reaction to stressors: The Cannon‘s “Fight or Flight” response (Cannon, 1935), and the Selye’s “General Adaptation Syndrome” (Selye, 1950 a; Selye, 1950 b).

The “Fight or Flight” reaction is mediated by the activation of the sympathetic-adrenomedullary system (SAS), with subsequent release of catecholamine hormones: norepinephrine from peripheral nerves and epinephrine from adrenal medulla (Reeder and Kramer, 2005). This response is usually prompt but brief, and determines (Figure 1):

i) Increase of heart rate and pulmonary ventilation;
ii) Vasoconstriction in skin and viscera;
iii) Vasodilatation in skeletal muscles and heart;
iv) Decrease of clotting time, to reduce blood loss consequent to injury;
v) Activation of catabolic processes, to provide energy to face increased requests from cardiovascular system, skeletal muscles, and brain.

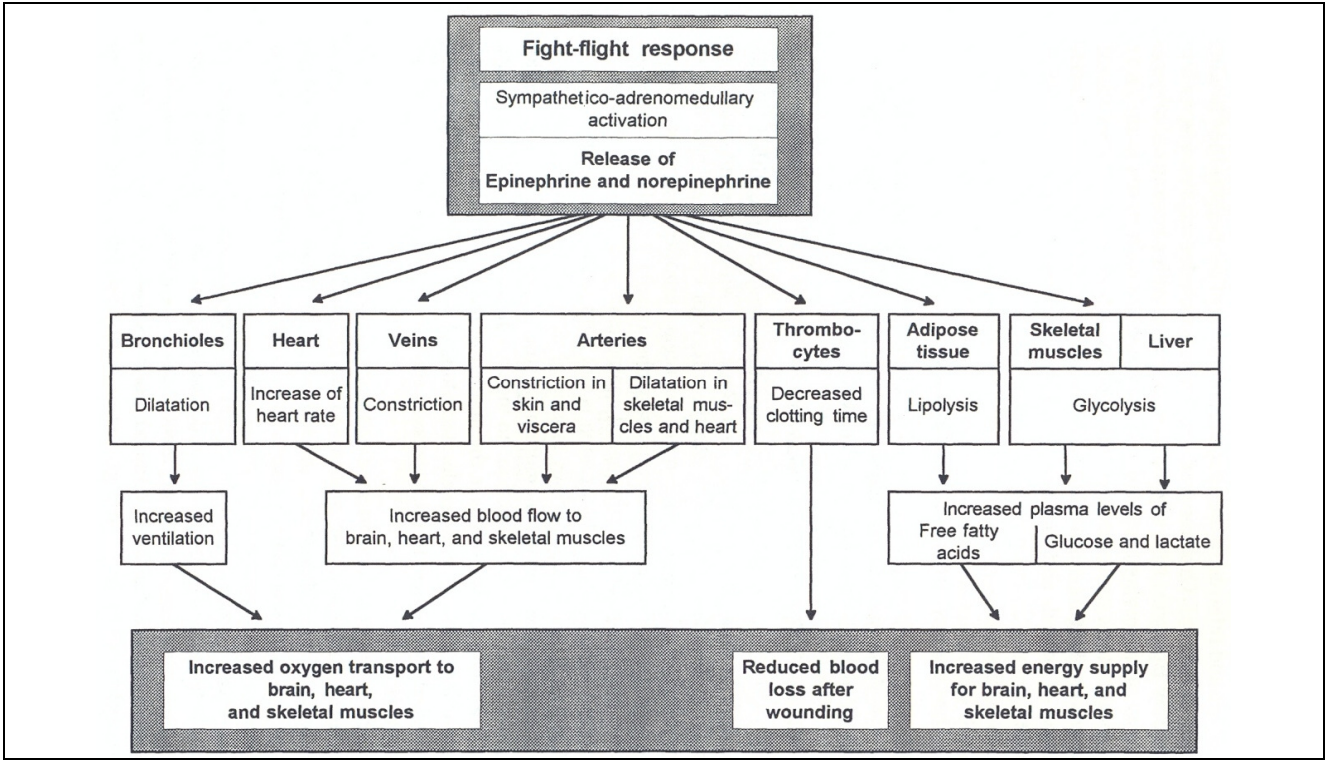

Figure 1: Cannon's "Fight or Flight" response: activation of sympathetic nervous system and release of adrenomedullary hormones (from: Von Holst, 1998).

Selye's "General Adaptation Syndrome" consists of three stages:

i) Alarm reaction: the hypotalamic-pituitary-adrenocortical system (HPA) is activated, with release of corticosteroids. If the stressor is too strong in this stage, death may result in few hours; but, if the stressor is not so strong and it is brief enough, the organism quickly regains its original state. According to McEwen and Wingfield (2003), it corresponds to the allostatic state: this state can be sustained for limited periods if food intake and/or stored energy (fat reserves) can fuel homeostatic mechanisms; if imbalance continues for longer periods, and becomes independent of maintaining adequate energy reserves, then symptoms of allostatic overload appear (McEwen and Wingfield, 2003).

ii) Stage of resistance: the organism adapts itself to the stressor, changing its physiological state. In this stage the activity of adrenal cortex is particularly important, and the gland enlarges its size to increase the hormones production.

iii) Stage of exhaustion: if the stressor is particularly strong or prolonged, the adaptation mechanisms fail, determining pathological state (gastro-enteric ulceration; immune depression; reproductive problems), till the organism dies (Selye, 1946). According to McEwen and Wingfield (2003), it corresponds to the allostatic overload. The cumulative result of an allostatic state is an allostatic load (i.e. the procedures that an organism implements to obtain food and survive and energy for extra needs, as migration, moulting, breeding); if additional loads of unpredictable events in the environment (e.g. disease, human

disturbance, social interactions) intervene, the allostatic load increases becoming allostatic overload.

Selye thought that any stressor could activate an aspecific response, but some years later Mason emphasized the importance of psychological components of stressors in determining different response patterns (Mason, 1968 a; Mason, 1968 b). Specific stressors can produce different neuroendocrine responses, either between or within different species, based on individual perception of the stressors and its capability to escape from them (Seligman, 1975).

Further studies (Henry, 1976; Henry and Stephens-Larson, 1985) defined that the stress response is determined by nature, duration, and intensity of the stimulus, as well as the perception of the individual.

In consequence of a stressor, behaviour may involve the animal moving away from the threat. If it is impossible, the animal will fight to cope the threat (active behaviour), or will hide or develop tonic immobility or other coping behaviour (passive behaviour) (Figure 2).

The activation of one of these responses depends on the perception of the individual: the active reaction is activated if the animal believes to have the possibility to cope with the stressor; on the contrary, if its reaction is supposed to be ineffective, the animal will use a subtractive response.

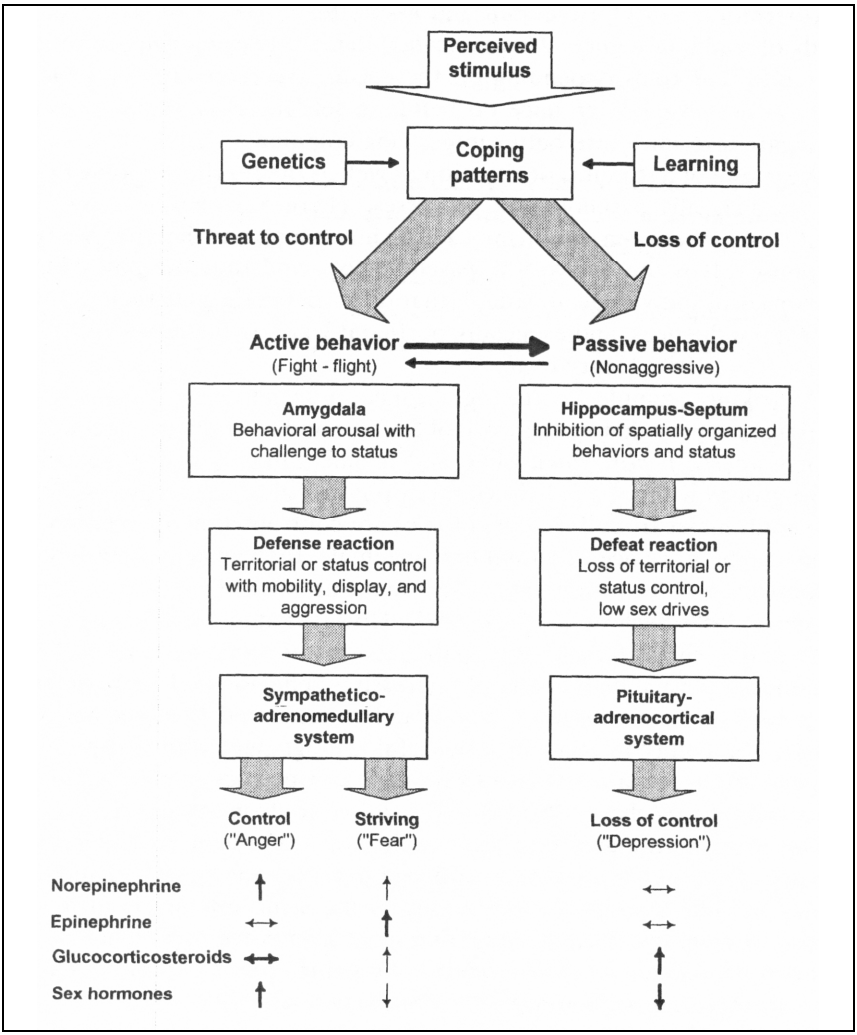

Figure 2: Schematic diagram contrasting the active and passive responses. The sympathetic-adrenomedullary system is divided into two branches: one of fight, anger, and norepinephrine; another of flight, fear, and epinephrine (from: Von Holst, 1998).

2.1.3 Hormones of stress

2.1.3.1 Catecholamine Hormones

When the sympathetic-adrenomedullary system (SAS) is activated, catecholamines are released and they can have important effects on metabolism: in particular, norepinephrine-noradrenaline is released from nerve fibres of locus coeruleus region (LUC-NE) in brainstem and epinephrine-adrenaline from adrenal medulla (Figure 3). The plasmatic concentration of catecholamines improves rapidly (few seconds after the action of the stressor), in consequence of a nervous mechanism, whereas their half-life is less than one minute.

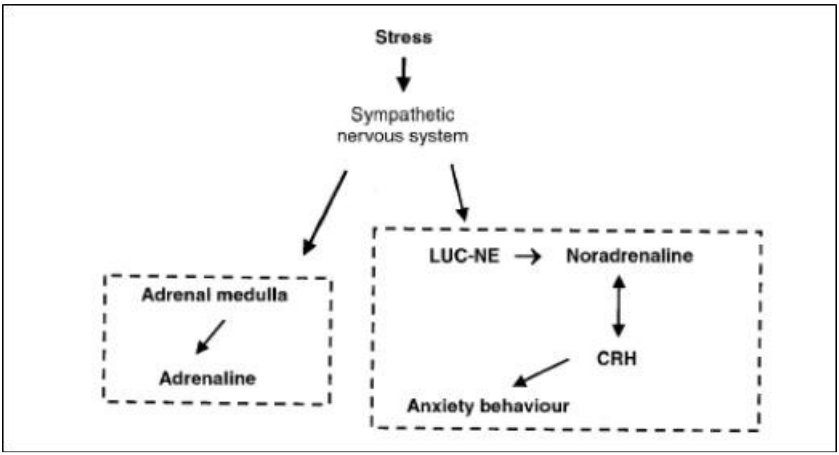

Figure 3: Sympathetic nervous system response to stress (from: Squires, 2003).

In consequence of catecholamines production, a series of physiological processes is activated, to increase available energy and organism reactivity (Figure 4) (Squires, 2003):

i) The glucose storage is inhibited and the release of glucose from the liver is activated: hyperglicemia;

ii) The fatty-acids storage is inhibited and the release of free fatty-acids from the liver and fat tissue is activated: arise of non-esterificated-fatty-acids (NEFA);
iii) The synthesis of protein is inhibited and the release of amino acids from muscles is activated;
iv) Improve of respiratory activity;
v) Splenic contraction;
vi) Direct effects on the cardio-vascular system: inotropic effect, peripheral vasoconstriction, arterial hypertension, increased blood flow to brain, heart, and skeletal muscles;
vii) Suppression of anabolic processes: growth, reproduction, and immune functions.

2.1.3.2 Glucocorticoids

Glucocorticoids are produced by the adrenal cortex owing to the stimulation from adrenocorticotropic hormone (ACTH), that is secreted by anterior lobe of pituitary gland (adenohypophysis) under the stimulus of the hypothalamic Corticotropin-Releasing Hormone (CRH) (Figure 4).

Arginine-vasopressin (AVP) has a very little ACTH secretagogue activity on its own. Glucocorticoids exert a negative feedback effect on the secretion of CRH, AVP, and ACTH, also inhibiting the suprahypothalamic centers controlling the HPA axis activity. This feedback activity is necessary to limit the exposure of tissues to glucocorticoids lipogenic, catabolic, anti-reproductive and immunosuppressive effects (Simpson and Waterman, 1995; Charmandari *et al*., 2004).

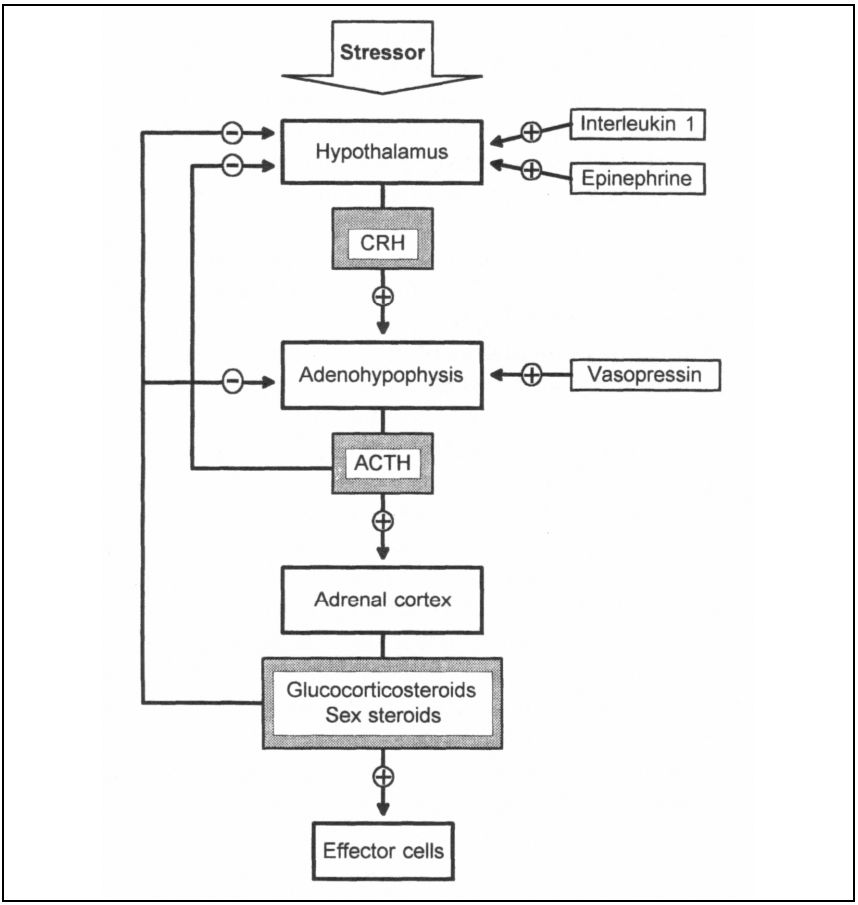

Figure 4: Schematic diagram of hypothalamo-pituitary-adrenocortical axis. Stimulating (+) and inhibiting (-) influences are indicated (from: Von Holst, 1998).

Principal glucocorticoids are cortisol and corticosterone. These hormones are produced in different proportions in different species: cortisol is predominant in man, cattle, sheep, dog, and pig; corticosterone in birds, rabbit, and rodents.

The increase of glucocorticoids determines metabolic modifications, as increase of gluconeogenesis, lipidic and proteinic catabolism, depression of reproductive and immune activity, and has direct activity on central nervous system (CNS).

Gluconeogenetic activity results from the glucocorticoids' capacity to induce the synthesis of glucose from non-glucidic precursors, especially proteins. In that way, available energy improves; but, muscular degeneration, reproductive problems and immunodeficiency can arise, if this action is too prolonged. In association with gluconeogenesis, an anti-insulinic activity of glucocorticoids can induce hyperglycemia.

Chronic activation of the HPA axis may induce negative effects on the organism (Charmandari *et al*., 2004):

i) Growth and development affections: growth hormone (GH) secretion is suppressed and insulin-like growth factor (IGF-I) is inhibited;
ii) Suppression of thyroid activity: thyroid-stimulating hormone (TSH) production is decreased; peripheral conversion of thyroxine to triiodothyronine is inhibited;
iii) Inhibition of gonadal function: CRH suppresses gonadotropin-releasing hormone (GnRH); glucocorticoids inhibits the GnRH neuron, pituitary gonadotroph and gonads, and renders target tissues of gonadal steroids resistant to these hormones (Figure 5) (Rivier and Rivest, 1991);
iv) Metabolic disorders: increased visceral adiposity, muscular degeneration, osteopenia/osteoporosis;
v) Immunosuppressive effects: glucocorticoids suppress cellular immunity and promote humoral immunity,

increasing the susceptibility to opportunistic pathogens (Figure 6) (Peterson *et al*., 1991; Apanius, 1998).

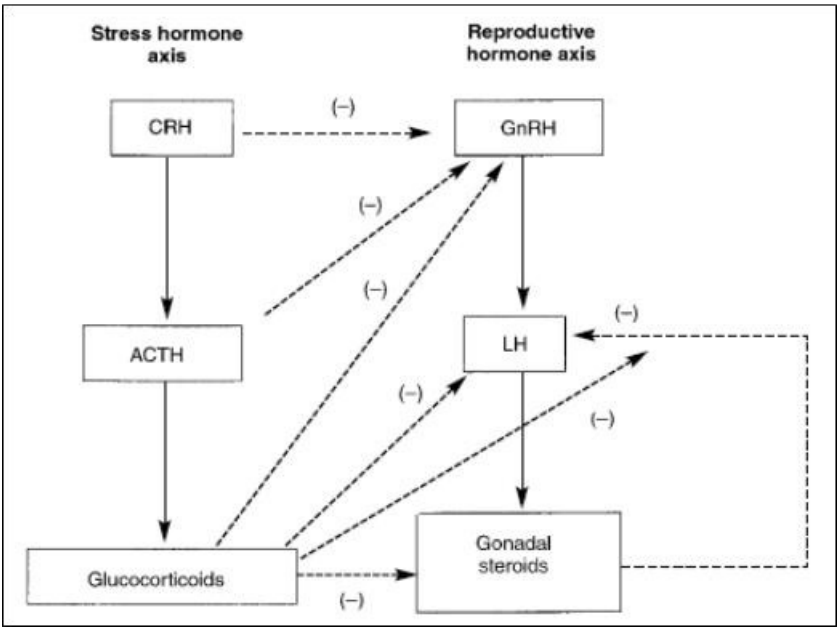

Figure 5: Effects of stress hormones on gonadal activity (from: Squires, 2003).

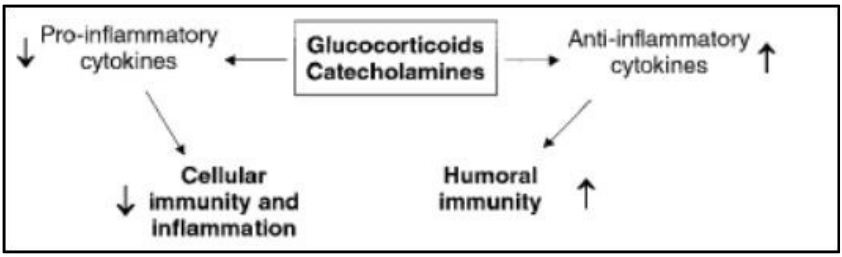

Figure 6: Effects of glucocorticoids and catecholamines on immune functions (from: Squires, 2003).

In Table 1 acute and potential long-term effects of glucocorticoids are summarized.

Acute effects	Chronic effects
Mobilization of energy (gluconeogenesis)	Loss of muscle mass, fatigue, steroid diabetes
Lipolysis (synergic with catecholamines)	Arteriosclerosis
Raised muscle contractibility (permissive to catecholamines)	Hypertension
Sodium retention and diuresis	Hypertension
Release of calcium from bones	Osteoporosis
Elevated release of hydrochloric and pepsinogen in stomach	Ulceration
Anti-inflammatory and immunosuppressive actions	Decreased wound healing, increased disease susceptibility
Suppression of gonadal activity	Decreased sexual behaviour, sterility
?	Dendritic atrophy (especially of hippocampal neurons)
Neural responses, including altered cognition and sensory thresholds	Psychoses and depression

Table 1: Acute and potential long-term effects of elevated levels of glucocorticosteroid hormones (modified from: Von Holst, 1998).

2.1.4 Acute and chronic stress

A stressor can be described qualitatively or quantitatively. “Quality” depends on its physical properties, whereas “quantity” depends on intensity and duration.

Intensity of a stressor can be precisely measured, but its impact on the homeostasis depends on the individual perception.

Duration permits to distinguish between acute and chronic stresses.

In wild animals, for example, capturing and handling a deer for radio-tagging is considered an acute stress: even if the intensity can be strong, the duration is usually brief (few minutes).

The permanence of snow for months in winter areas can be considered a chronic stress, because under these conditions the animals movements and food search are made difficult for long periods (Shackleton and Bunnell, 1987; Von Petrak, 1993). A practical example of chronic stress is the building of a dam in 1980-81 on the Rocky Mountains, with increased human contacts, vehicular traffic, atmospheric dust and noise, that determined a die-off of bronchopneumonia in bighorn sheep (*Ovis c. canadensis*, Shaw), with a loss of 75 to 85 % of the sheep (Spraker *et al*., 1984).

When an acute stress is repeated for a long period, it can be defined as chronic intermittent stress (Burchfield, 1979; Ladewig, 2000), such as, for instance, the disturbance of snowmobiles in winter areas (Creel *et al*., 2002).

If an acute stress is repeated for a period, the HPA system can vary its activity over time: the response can increase in intensity (sensitization), decrease in intensity (desensitization), or have no variations (Ladewig, 2000). It depends on different factors: generally,

a highly intense stressor is more likely to result in sensitization than a stressor of low intensity (Konarska *et al*., 1990). Furthermore, desensitization is stressor-specific: the desensitization to a specific repeated stressor does not extend to a different (heterotypic) stressor (Kant *et al*., 1985).

2.1.5 Welfare

The first interest for animal welfare arose after the publication of Ruth Harrison's "Animal Machines", a book about intensive animal productions, that shocked the public opinion (Harrison, 1964).

Consequently, in 1965 the British Government published the Brambell Report (Brambell Report, 1965), on the welfare of farm animals, in which the "five freedoms" were enunciated for the first time:

i) Freedom from thirst, hunger, and malnutrition;
ii) Freedom from thermal, and physical discomfort;
iii) Freedom from pain, injury, and disease;
iv) Freedom to express normal behaviour;
v) Freedom from fear, and distress.

In few years, these indications were acknowledged from European Strasburg Conventions (1976 and 1979), and from several National Legislations, and revised from the Farm Animal Welfare Council (FAWC, 1993).

2.1.6 Definition of welfare

Welfare must have a precise meaning, due to its use in science and legislation (Broom, 1991 b). The definition should be comprehensible for different categories of people: consumers, veterinarians, politicians, corporations, and others (Hewson, 2003). Welfare refers to a characteristic of an individual, rather than to something given to it; it

must be measurable in a scientific way; and it can vary over a scale from very good to very poor (Broom and Johnson, 1993; Curtis, 1986; Duncan, 1987; Broom, 1988; Broom, 1991 a; Broom, 1996).

Along the time, several definitions of welfare were given, according to the perception of animals status and human-animal relationships in different periods or situations.

The first definitions related mainly to physical and environmental conditions, neglecting psychological aspects (Blood and Studdert, 1988). Measurements of welfare also focused on physiological parameters (plasma, urine, faeces cortisol, heart rate, blood pressure, weight changes). The problem is that genetics and environment can improve some animal aspects (e.g. productivity), maintaining in some cases a compromised mental status. Furthermore, physiological measures can vary both in positive and negative situation (Hewson, 2003).

Today, the organism is considered in a more comprehensive way, as the whole of body and mind: in this case, to assess animal welfare it is necessary to measure behavioural signs.

On scientific basis, three main approaches have been attempted to define welfare, stressing different aspects (Hewson, 2003; Carenzi and Verga, 2009):

i) The importance of the biological functions of the organism (growth, reproduction, health): in this case, welfare is considered as the response to environmental changing. The definition of Broom can be included in this approach: he defined the welfare of an individual as "its state as regards its attempts to cope with environment"

(Broom, 1986). In this case, the concept of welfare is something of dynamic because of the continue environmental conditions variations.

ii) The relationship between stress and welfare: it was acknowledged already from Brambell Report that "welfare is a wide term that embraces both the physical and mental well-being of the animal. Any attempt to evaluate welfare, therefore, must take into account the scientific evidence available concerning the feelings of animals that can be derived from their behaviour". In 1976 Huges gave this definition: "Welfare on a general level is a state of complete mental and physical health where the animal is in harmony with its environment" (Huges, 1976). This is very similar to the definition of World Health Organization, for which welfare is "a state of complete physical, mental and social well-being, and not, merely the absence of disease or infirmity" (WHO, 1946). According to Curtis (1985), in order to ensure animal welfare, "the fulfilment of all the animals' physiological, safety and behavioural needs is required". Also in this case, welfare is dynamic, but the motivation is that physiological and behavioural needs vary continuously along the time. Curtis proposed a hierarchic organization of animal needs, based on priority, as in the human model by Maslow (1962), with physiological needs at the base (high priority) and behavioural needs at the vertex (low priority). In this approach, care must be taken in order to avoid anthropomorphic interpretations (Morton *et al*., 1990).

iii) The possibility for an animal to live according its natural attitudes and behaviour: in this case the problem is that

domesticated animals are very different and they have different features from the wild ones (Price, 1984).

Recently, the most widely accepted definition of animal welfare is that it "comprises the state of the animal's body and mind, and the extent to which its nature (genetic traits manifest in breed and temperament) is satisfied" (Duncan and Fraser, 1997), even if the three aspects often can conflict, determining practical and ethical problems.

In wildlife conservation biology, welfare-related arguments were typically avoided, considering them irrelevant. Actually, even from a completely practical perspective, welfare science should be applied in conservation biology, even not considering animals like sentient individuals: for example, welfare is significant in reaching conservations aims, as reintroduction and relocation of endangered species (McLaren *et al*., 2007).

2.1.7 Measurement of stress and welfare in wild animals

Measurements of stress and welfare are frequently employed in controlled animals, in controlled conditions: this allows to measure environmental conditions, and to have accustomed animals that are not frightened by man, reducing the bias due to sampling. In spite of that, some methodological problems are still present, also in domestic and laboratory animals:

i) Animal housing and handling: any handling of animals functions as a stressor that acts on the corresponding variables within seconds (catecholamines), minutes (glucocorticoids, thyroid and gonadal hormones), or few hours (some immunological parameters) (Von Holst, 1998; Beerda *et al*., 1996; Carlstead *et al*., 1992; Willemse *et al*., 1993);

ii) Individual, circadian, seasonal and other variations of physiological parameters: the secretion of hormones is not continuous, but occurs in a pulsatile mode, and hormones concentration in blood can vary by a factor 10 or more within minutes (Von Holst, 1998; Ingram *et al.*, 1999; Lynch *et al.*, 2002).

In wildlife, some attempts have been done, but there are some difficulties in addition to those mentioned above:

i) Wild animals fear man much more than domestic or laboratory ones: when we need to capture a wild animal for sampling, each manipulation may become a source of stress, that can misrepresent the actual situation (e.g., corticosteroids concentration varies in only 3 minutes after the beginning of blood sampling procedures).
ii) Environmental conditions are often very hard, and sometime it is impossible to collect samples in a correct way.
iii) When a wild animal is captured, we usually don't know its life-history.
iv) Sampling in wild animals is often, done on culled individuals, because of the relative facility to have access to carcasses during the hunting season: this implies that it is impossible to have repeated information about the same individual.

It is important to note that SAS and HPA system can be activated both in beneficial and in detrimental situations: so care must be taken in interpretation of the results.

In Table 2 and Table 3, the principal methods and indicators for assessing HPA activity and SAS activity, with their relative advantages and disadvantages, are summarized. The principal methods and indicators for assessing welfare are reported in Table 4.

Method/Indicator	Advantages	Disadvantages
Adrenal gland weight and relative adrenal weight (adrenal weight/animal weight) (Christian, 1963; Bronson and Eleftheriou, 1964; Adams and Hane, 1972; Sauerwein *et al*., 2004)	Useful in smaller animals in the wild	Animals have to be killed
	The relative one should compensate for differences in animals size	It is not possible to follow response of adrenocortical system in animals on the individual level
		No information on short-term changes in adrenal activity (changes in size request several days of stress)
		In stressed animals weight changes quickly and it can conditions the ratio
		Increased adrenal weights can be determinate by genetic background and metabolic status (Wise *et al*., 1993)
Histology of adrenal gland (Sauerwein *et al*., 2004)		Indirect method: it was used in the past, but now it is dropped
Concentration of glucocorticosteroids (GCS) in blood (Rehbinder and Hau, 2006; Del Giudice *et al*., 1990; Bateson and Bradshaw, 1997; Morrow *et al*., 2000)	Relatively simple	GCS concentration varies in 3 minutes after the beginning of blood sampling procedures
	Less than 10 µl of blood are needed: also suitable in small animals and for repeated sampling	Applicable only in controlled laboratory conditions
		Most of studies determine the total amount of GCS (protein-bound=inactive and protein-unbound=active)
Concentration of free-GCS in urine (Rehbinder and Hau, 2006; Trindle, Lewis and Lauerman, 1979; Del Giudice *et al*., 1990; Morrow *et al*., 2000; Saltz and White, 1991)	Relatively simple	Time lag in GCS
		GCS concentration in urine changes during the day: the daily urine production should be needed
		The concentration of hormones in the urine varies according the amount of urine produced (urine production and water assumption are conditioned by stress)

(Table 2: continued)

Method/Indicator	Advantages	Disadvantages
Concentration of free-GCS in faeces (Millspaugh and Washburn, 2004; Rehbinder and Hau, 2006; Möstl *et al*., 2002 a; Palme, 2005; Millspaugh and Washburn, 2003; Dehnhard *et al*., 2001; Coburn *et al*., 2010; Chelini *et al*., 2006; Washburn and Millspaugh, 2002; Möstl and Palme, 2002 b)	Relatively simple	Sampling must be done immediately after faeces deposition: air, temperature, rain and other factors may influence GCS concentration in faeces (Millspaugh and Washburn, 2004)
		Conservation requires freezing temperature
		GCS concentration in faeces changes during the day: the daily faeces production should be needed
		Time lag in GCS
		The concentration of hormones in the faeces varies according the amount of faeces produced
Concentration of free-GCS in saliva (Rehbinder and Hau, 2006; Menargues *et al*., 2008)	It corresponds in most species to the concentration in blood	
	It is independent from saliva production	
	Very fast	
	It is needed about 1 ml of saliva	
	It measures only the active protein-unbound fraction of the hormones	
Concentration of CGS in hair (Koren *et al*., 2002; Davenport *et al*., 2006; Accorsi *et al*., 2008; Bennet and Hayssen, 2010; Macbeth *et al*., 2010; González-de-la-Vara *et al*., 2011; Comin *et al*., 2011; Comin *et al*., 2012 a; Comin *et al*., 2012 b; Montillo *et al*., 2012)	Non-invasive	No information available for wildlife
	Fast and easy sampling and conservation	
	It is needed just few mg of hair	
	It is possible to determine chronic stress and to temporize it, cutting hair at different length	
Leukocyte profiles (Davis *et al*., 2008)	They are altered by stress and can be directly related to stress hormone levels	
	GCS determine neutrophilia and lymphocytopenia	
	In wild ruminants neutrophilia and lymphocytopenia can occur within 1 hour (López-Olvera *et al*., 2007)	
	Low cost	

Table 2: Principal methods/indicators for assessing HPA activity, with their relative advantages and disadvantages.

Method	Advantages	Disadvantages
Concentration of catecholamines in blood (Kvetnansky *et al.*, 1970; De Boer *et al.*, 1989)	Relatively simple in controlled situation	Because of capturing and handling animals activates in few seconds the SAS, it is impossible, to sample blood without prior introduction of cannulas into the blood vessels (Stoddard, 1991)
Heart rate (Harms *et al.*, 1997; Butler, 1993)	Relatively simple in controlled situation	It is necessary an implantable radio transmitter to record heart rate telemetrically: this can be a problem in wild animals because of the implantation, the range of transmitter system, and the working life of the transmitter
		Wild animals are usually under the effect of narcotics that can influence the heart rate
Blood pressure (Herd *et al.*, 1969; Lamprecht *et al.*, 1973)	Relatively simple in controlled situation	Animals must be handled and restrained
		Wild animals are usually under the effect of narcotics that can influence the blood pressure

Table 3: Principal methods/indicators for assessing SAS activity, with their relative advantages and disadvantages.

Method/indicator	Advantages	Disadvantages
Body weight change (McLaren *et al*., 2004)	Relatively easy	Body weight can vary for a number of causes (food supply, reproductive period, lactation, sex)
	Animals can be weighted with non-invasive techniques in particular conditions (Bassano *et al*., 2003)	In most cases, animals need to recaptured in order to be re-weight, causing further stress
Breeding success Direct measures: litter size, young weaned in mammals; clutch size, hatching success, and fledging in birds (Clinchy *et al*., 2004) Indirect measures: number of nests, number of visits to the nest, adults in an area (Anderson and Keith, 1980)		Difficult to assess in wild animals in most cases (anthropic disturbance)
Life expectancy (Pickering, 1989; Hurnik and Lehman, 1988)		Difficult to assess in wild animals
Heart rate (Harms *et al*., 1997; Butler, 1993)	Useful in short term	Not useful in long term conditions
		See Table 3
Blood pressure (Herd et al., 1969; Lamprecht *et al*., 1973)		See Table 3
Haematocrit and haemoglobin levels changes (Upton and Morgan, 1975)	Useful in short term	Animal handling is needed
Respiratory rate (Silanikove, 2000; Fraser and Broom, 1990)	It can be assessed by observation of a stationary animal from distance	Mainly used in experimental animals (Morton and Griffiths, 1985)
	It is regularly measured in captured wild animals	
Body temperature (Moe and Bakken, 1997)	It is regularly measured in captured wild animals	Body temperature varies physiologically during the day
		Handling animal is needed
		Some events can increase core temperature, but decrease peripheral temperature due to vasoconstriction

(Table 4: continued)

Method/indicator	Advantages	Disadvantages
Quality of meat (muscle and other carcass characteristics) (Gregory, 1998)		Animal have to be killed
Behaviour (Rutherford, 2002; Dawkins, 2004)	It is a precocious index: usually, behavioural changes are the first attempt to cope with environmental difficulties (Broom and Johnson, 1993)	It is necessary to know specie-specific behaviours and normal behaviour of the animals (Squires, 2003)
	Non-intrusive	
	Non invasive	

Table 4: Principal methods/indicators for assessing animal welfare (short- and long-term responses), with their relative advantages and disadvantages.

2.2 Body condition and nutritional status

In wildlife management, nutrition plays a central role in recruitment and loss in animal population (Sinclair *et al.*, 2006), since food abundance and distribution can directly influence emigration, immigration, and mortality (Harder and Kirkpatrick, 1996).

The nutritional status of an animal is related to its fitness (i.e. reproductive success), and its implications are of great interest to ecological studies (Green, 2001): Individuals must collect sufficient nutrients to cover their energetic requirements and to provide a surplus large enough to allow them to reproduce (Clutton-Brock *et al.*, 1982 a).

Animal's health is an indicator of the past foraging success, fighting ability, and the ability to cope with environmental pressures (Jakob *et al.*, 1996). For example, in ungulates the body mass is a determinant of juvenile survival in roe deer (*Capreolus capreolus*) (Gaillard *et al*., 1997) and mule deer (*Odocoileus hemionus*) (White *et al*., 1987); adult survival in big horn sheep (*Ovis canadensis*) (Bérubé *et al*., 1999); litter size in roe deer (Hewison, 1996); and age at first breeding in red deer (*Cervus elaphus*) (Albon *et al*., 1987; Carrion *et al*., 2007) and roe deer (Gaillard *et al*., 1992; Loudon, 1987; Hewison, 1996).

2.2.1 Body condition and nutritional status in wild ungulates

Information on the body condition of ungulates can reveal much about nutritional history of the animals: measuring enough animals in a population can give information about the general health status of the population, and its response to environmental quality (Adamczewski, 1993).

In ungulates, we assist to complex seasonal changes in weight and physiology, due to environmental and endogenous cycles guided mainly by photoperiod. In the past, it was simply thought that the animals grew and fattened during the summer, due to the food abundance, and they lost weight during the winter, due to reduced food resources: the fat reserves accumulated during the summer were thus necessary to overwinter (Adamczewski, 1993).

Actually, in temperate habitats, metabolic rate and food intake in white-tailed deer (Silver *et al*., 1969; Holter *et al*., 1976; Short, 1975), red deer (Kay, 1978), reindeer (McEwan and Whitehead, 1970), and moose (Gasaway and Coady, 1974) decline during winter, probably to reduce the energetic costs of feeding in hard situations, and that occurs also in captive deer with free access to food supplementation during the winter: they show a seasonal cycle in food intake and weight loss similar to that in the wild, even if less extreme. Seasonal changes in weight are not only related to fat, but also to protein, in particular skeletal muscles (Adamczewski, 1993). Seasonal cycles in weight vary widely among sex and age classes, populations and years: adult males reach the peak just before the rutting period and lose weight in short time. Adult females reach the peak later and tend to lose weight in late winter. During the winter, the weight loss is evident in wild deer of different classes, and the growth of the young is slowed (Adamczewski, 1993).

As an example of seasonal changes in energy demand, rough estimated energy requirements for red deer are showed in Figure 7: In pregnant and lactating females, both energy and protein requirements increase logarithmically during the last third of gestation (Mohen, 1973; Verme, 1965), and in a more manifest way during lactation, peaking during the fourth to sixth week after parturition (Mohen,

1973; Mohen, 1978). In stags, during the rut, the metabolic requirements are estimated to be greater than those of hinds at the lactation peak.

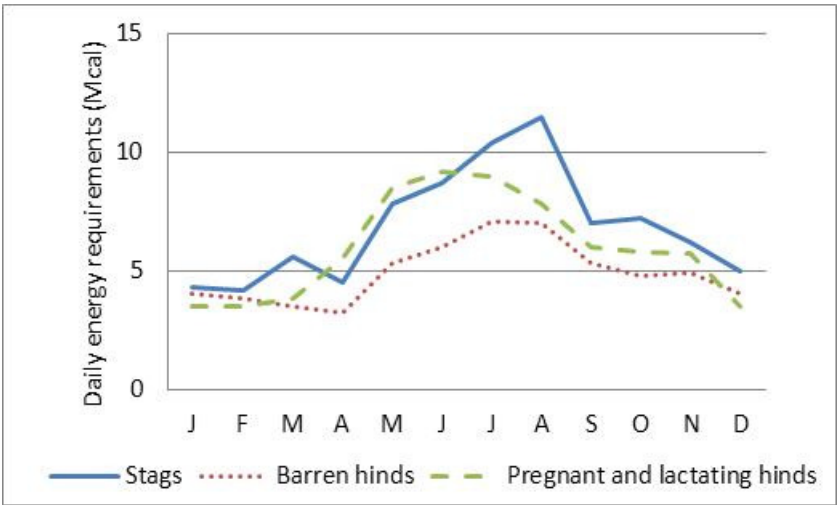

Figure 7: Estimated daily energy requirements of red deer stags, barren hinds, and pregnant and lactating hinds in different months (January-December) (Anderson, 1976). Estimates represent only a rough approximation of the energy requirements of the three classes for red deer (modified from: Clutton-Brock *et al.*, 1982 a).

2.2.2 Assessment of body condition and nutritional status in wild ungulates

To assess body condition and nutritional status of wild ungulates, three time scales can be used (Adamczewski, 1993):

i) Recent (days or week), indicative of nutrition and health (nutritional status): measures from blood, urine, faeces, and gut contents;

ii) Intermediate (months or season), indicative of animals' body reserves (body condition): body weight, measures of fat and protein reserves;

iii) Long-term (years), indicative of population quality in its habitat: linear body measurements and bone lengths.

Measuring nutritional status and body conditions is more significant when used broadly, and together with other information on population characteristics, diseases, and range quality.

Timing of data collection can give different information: measuring body condition in the early winter gives a cumulative measure of summer/fall nutrition; in the late winter, nutritional stress is more severe (Adamczewski, 1993).

Indices are species- and sex-specific; they can also vary seasonally and between different populations (Miller, 1989; Sparling *et al*., 1992). So care must be taken in applying body condition indices without confirming their predictive value in a particular species (Gleixner and Meyer, 1997; Schamber *et al*., 2009).

Some measures of nutritional status and body condition can be collected only from dead animals (culled or found dead), below defined "Invasive methods/indicators", whereas others can be collected also on live animals (free-ranging or captured), below defined "Non-invasive methods/indicators". The principal methods used to assess body condition and nutritional status are summarized in Table 5, Table 6, Table 7 and Table 8.

Method/indicator	Advantages	Disadvantages
Kidney Fat (Riney, 1955; Nieminen and Laitinen, 1986)	Good correlation with the animal's total fat if measured broadly in a population	Variability among users: a training is needed to avoid that they collect only part of the kidney fat
Kidney Fat Index (KFI) (Riney, 1955; Holand, 1992)	Good correlation with the animal's total fat if measured broadly in a population	Variability among users: a training is needed to avoid that they collect only part of the kidney fat
		Kidney weight can vary widely among seasons
Carcass weight (weight of the entire animal except for gut contents) (Mitchell *et al*., 1976; Adamczewski *et al.*, 1995; Ichimura *et al*., 2004)	It is not affected by the weight of gastro-enteric content	Not a valid indicator for comparison of nutritional status among populations with different genetic background
	It can be used also in hunted animals	
Fat content of the marrow of long bones (Mech and Delgiudice, 1985; Nieminen and Laitinen, 1986; Holand, 1992)	It provides a sensitive measure of fat depletion in its final stages	Little variation in depletion stages other than the final one
Depth of back fat (Hughes *et al*., 2009)	Good correlation with the animal's total fat	Variability among users
	Quick	
	Non-invasive if ultrasounds are used (Maximini *et al*., 2012)	
Weight of individual muscles (e.g. gastrocnemius) (Adamczewski, 1993)	Reliable index of skeletal muscle weight and body protein	
Appearance of fat on heart, omentum, kidneys, brisket, tailbase, and on fullness of muscles (Kistner *et al*., 1980)	Good indication of body condition	Variability among users
		Sample collection is difficult

Table 5: Invasive methods/indicators to assess body condition in wild ungulates.

Method/indicator	Advantages	Disadvantages
Total body electrical conductivity (TOBEC) (Snyder *et al*., 2005)		In ruminants, water in gastro-intestinal tract can determine variability in the results
		30 minutes of sampling are needed to obtain the result: it is difficult in field condition
Ultrasound measurement of fat thickness (Cook *et al*., 2001 a; Cook *et al*., 2001 b; Stephenson *et al*., 1998; Stien *et al*., 2003; Gustine *et al*., 2007)	Extremely precise	Equipment expensive
		It can only measure subcutaneous fat
		It has been little tested in wild animals
Total body mass (live animals) (Pettorelli *et al*., 2002)	Usually correlated with condition, within sex/ages classes	Gut contents can be an unknown variable
		Hard to measure in field in large animals
Body condition score (Audigé *et al*., 1998; Cook *et al*., 2004)	Easy and rapid	Variability among users
Hind foot length (Toïgo *et al*., 2006; Zannèse *et al*., 2006; Garel *et al*., 2010)	It is a relevant indicator of phenotypic quality	Variability among users
	It is easy to collect over large scale	
	It is not conditioned by gut contents	

Table 6: Non-invasive methods/indicators to assess body condition in wild ungulates.

Method/indicator	Advantages	Disadvantages
Rumen contents and faeces (Anthony and Smith, 1974; Holechek *et al*.,1982; Gebert and Verheyden-Tixier, 2001)	Good index of recent diet quality	It provides only a broad indication of diets, because some plants and plant parts disappear rapidly from the rumen
Enlargement of rumen papillae (Hofmann, 1982)	They enlarge and increase their surface area rapidly in response to greater fermentation in the rumen and in correlation with rate of nutrient absorption in the rumen	
Weight of several organs and glands (liver, kidney, thyroid, thymus) (Mitchell *et al*., 1976)	Easy	Some organs and gland show pronounced seasonal weight variation

Table 7: Invasive methods/indicators to assess nutritional status in wild ungulates.

Method/indicator	Advantages	Disadvantages
IGF-1 (Insulin-like Grow Factor) (Suttie *et al*., 1993; Bubenik *et al*., 1998)	It is highly correlated with rate of lean body growth	It is affected by season, reproductive status, disease
	It is very sensitive to nutritional changes	It requires specialized laboratory assays
	Its blood levels are stables	
T3 (Triiodothyronine) (Delgiudice *et al*., 1987)	It is correlated with metabolic rate and reduced following malnutrition	It is affected by season, reproductive status, disease
	Its blood levels are stables	It requires specialized laboratory assays
Glycaemia (Jenks *et al*., 1991; Klein *et al*., 2002)	Easy and fast	It can vary due to anaesthetic use
		It is instable in blood: the measure need to be taken immediately after sampling
Plasma Urea/Creatinine ratio (Milner *et al*., 2003)	It is relatively sensitive to recent protein intake, so correlates with diet quality	Its levels can be affected by season and non-nutritional stress
	Its blood levels are stables	High levels may reflect good food or increased protein catabolism
Urinary Urea/Creatinine ratio (Warren *et al*., 1982)	It is relatively sensitive to recent protein intake	Its levels can be affected by season and non-nutritional stress
	It can be used to identify severe malnutrition	High levels may reflect good food or increased protein catabolism (in this case: hyperpotassemia)
Diet composition assessed from faeces (Beier, 1987)	It is indicative of short term diet	Expensive: specialized operators are required
Fecal Nitrogen Index (Leslie and Starkey, 1985)		

Table 8: Non-invasive methods/indicators to assess nutritional status in wild ungulates.

2.3 Reproduction

In population dynamics, reproductive performances occupy an important position: the ability to produce healthy calf crop will replace the losses due to mortality factors such as predators, hunters, vehicle collisions, disease, poachers, and winterkill (Stelfox and Stelfox, 1993). Selection pressures will favour mothers that minimize calf mortality: hinds must minimize predators effect, at the same time ensuring a rapid growth of their calves, that must be healthy and strong enough to overwinter (Clutton-Brock *et al*., 1982 b).

Monitoring reproductive parameters (such as sexual maturity, age at first breeding, cow/calf ratio, calf survivorship, calving interval) indicates how populations respond to human or environmental influences (Stelfox and Stelfox, 1993).

2.3.1 Factors affecting female fecundity in deer

Many factors can affect fecundity in female deer. For example, the following factors have been recorded in red deer hinds (Clutton-Brock *et al*., 1982 c):

i) Age at first breeding: it is due to population density and nutritional status (see Chapter 2.2).
ii) Differences in body weight: it is due to differences in body condition.
iii) Available resources in the mother's home range: the quality and quantity of available resources influences the body condition.
iv) Population density: in deer, daughters adopt home ranges overlapping with those of their mothers, thus fecundity can decrease as the number of resident daughters increases, due to the competition for food (Clark, 1978).

2.3.2 Factors affecting calf mortality in deer

Variation in the frequency of calves mortality is the most important source of differences in reproductive success in hinds (Clutton-Brock *et al*., 1982 c). According to Clutton-Brock, in red deer on the Isle of Rum (Scotland), on average, 20% of calves die before the end of September following their birth, and a further 11% die during the following winter. In Italy, calves mortality in the three months after birth is about 5% (Mattiello and Mazzarone, 2010).

Several can be the causes of calves mortality (Clutton-Brock *et al*., 1982 c):

i) Stillborn calves.
ii) Problems of sucking or milk shortage in the mother.
iii) Accidents.
iv) Calves deserted or killed by mother.
v) Predation.
vi) Malnutrition, often worsened by parasite infestation.
vii) Low birth weight: influenced by mother's weight and condition in spring.
viii) Birth date, influenced by mother's conditions during the rut: late born calves are lighter in autumn/winter than early-born ones and may die more frequently for this reason.
ix) In red deer, male calves and yearlings born in large matrilineal groups tend to have higher mortality than those born in small matrilineal groups, probably due to ecological factors affecting the calves after birth.

2.3.3 The oestrous cycle and the sexual hormones

In most species, in the absence of pregnancy, the mature female undergoes a continuous series of reproductive cycles, in which a

group of ovarian follicles matures (follicular phase), the female becomes receptive to mating (oestrous or heat), and the dominant follicle ovulates and a corpus luteum is formed (luteal phase) (Figure 8).

The number of follicles ovulated and corpora lutea formed is characteristic of the species. If fertilization and pregnancy does not occur, the corpus luteum regresses (luteolysis) and the cycle repeats itself.

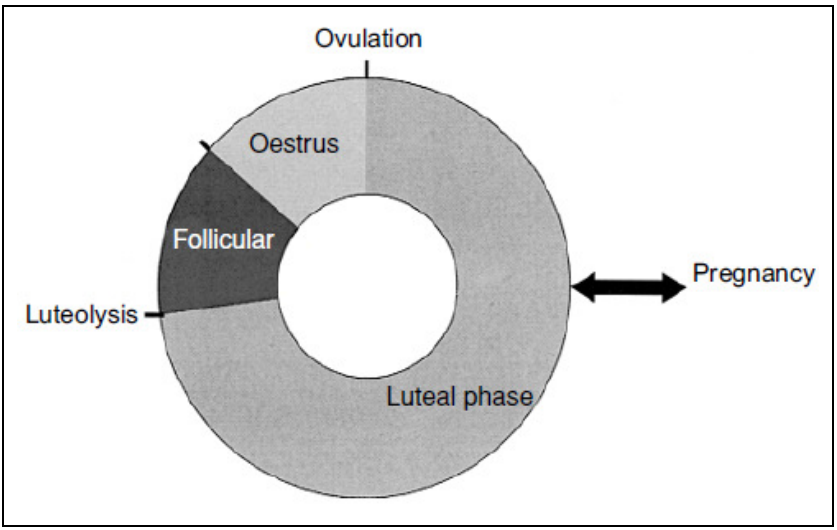

Figure 8: Outline of the oestrous cycle (Squires, 2003).

Hypothalamus releases, via hypothalamic-hypophyseal portal blood vessels, the Gonadotrophin Releasing Hormone (GnRH), that acts on the anterior pituitary, driving the release of gonadotrophic hormones: Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), acting on the ovary and determining the maturation of ovarian follicle (Figure 9).

Both GnRH and gonadotrophins are released in a pulsatile manner, thus affecting biological function. Several external factors influence the pulse frequency: nutrition, stress, suckling, presence of males (pheromones), season, visual and olfactory stimuli (Figure 9).

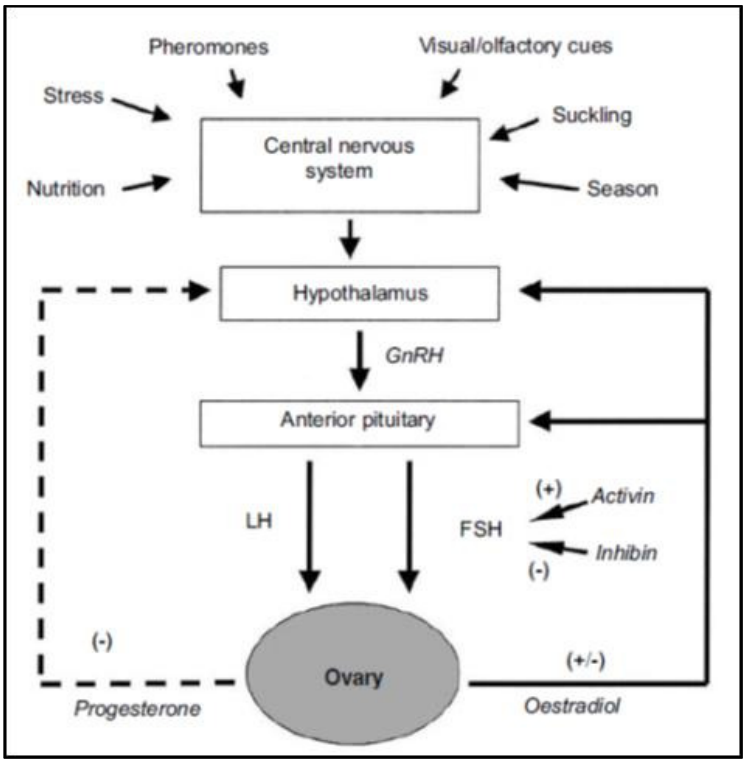

Figure 9: Hormonal regulation of the oestrous cycle (from: Squires, 2003).

Inhibin and activin are produced by the gonads and regulate the release of FSH from the pituitary: inhibin reduces the production of FSH, activin increases the production of FSH, independently from GnRH.

Several primary follicles are contained in a mammalian ovary at birth: during the life, some of them will gradually mature, whereas most of them will undergo atresia. Maturation is driven in particular by FSH and LH. When a primary follicle becomes a dominant one, it produces oestradiol which acts on the brain, inducing the oestrous behaviour, increasing LH production and decreasing FSH production: this leads to a surge in LH release that culminates in rupture of the follicle, release of the ovum (ovulation) and formation of the corpus luteum on the ovary. It is important to note that elevated levels of cortisol can block or delay the pre-ovulatory LH surge.

The cells of corpus luteum produce progesterone, which inhibits GnRH secretion, and thereby decreases pulsatile LH release: so the presence of a functional corpus luteum prevents ovulation during the luteal phase.

Oestrogen acts on the uterus to increase the number of receptors of oestrogens and oxytocin. If fertilization and implantation do not occur, high levels of progesterone and oxytocin from the ovary stimulate the uterus to secrete prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ causes regression of the corpus luteum by interfering with LH action on the corpus luteum and increases the production of the oxytocin by the ovary.

Progesterone is necessary for the maintenance of pregnancy and it is produced by the ovary, placenta and adrenal gland. The relative importance of these sources depends on the species and on the stage of gestation. High levels of progesterone and oestrogen reduce GnRH release from hypothalamus and LH release by the pituitary (Squires, 2003).

2.3.4 Oestrus cycle and pregnancy in red deer

In red deer from temperate climate, in good environmental conditions, hinds reach puberty at about 15-18 months (Kröning and Vorreyer, 1957; Ratcliffe, 1984; Mattiello and Mazzarone, 2010). In high density condition, hinds could reach puberty at about 28-40 months (Mitchell *et al*., 1977) or more (Mattioli, 1993). The reproductive capacity in hinds is connected to the reach of the threshold of about 70-75% of the adult weight (Kelly and Moore, 1977; Albon *et al*., 1983; Albon *et al*., 1986; Langvatn *et al*., 1996; Mattiello and Mazzarone, 2010).

Deer are a seasonal polyestrous species: in the Northern hemisphere, the onset of the mating season is usually in September-October and, in the absence of pregnancy, hinds can show up to 8 successive cycles, with the last oestrous in March. The mean length of an oestrous cycle is 18-19 days (Guinness *et al*., 1971; Kelly *et al*., 1985), although there is a tendency for cycle length to decrease with increasing age of hinds, and to increase as the mating season progresses. Sometimes cycles of 7-9 days, or longer than 30 days are observed. The oestrous length is about 12-24 hours and it tends to be synchronized in hinds of the same herd (Guinness *et al*., 1971; Iason and Guinness, 1985).

Plasmatic levels of progesterone in peri-oestral period in red deer are described as low (less than 1 ng/ml) (Adam *et al*., 1985) or variable up to 4.7 ng/ml (Adam *et al*., 1985): variable and elevated levels of progesterone during oestrus could be due to accessory corpora lutea (Guinness *et al*., 1971; Kelly and Challies, 1978), or to a persistent corpus luteum or could have origin from adrenal gland (Thimonier and Sempere, 1989). Plasmatic levels of progesterone are exceeding 1 ng/ml from the 4th day after oestrus, reaching higher levels (3-15

ng/ml) in the second half of the luteal phase, and reducing drastically with luteolysis (Thimonier and Sempere, 1989).

A LH pre-ovulatory peak can be detected in red deer (Kelly *et al*., 1985). Between the end of the luteal phase and the oestrus, oestrogens (in particular oestradiol) reaches high levels (in red deer, 120 pg/ml) (Kelly *et al*., 1985). In red deer usually just one follicle ovulates: if fertilization and pregnancy does not occur, the corpus luteum regresses (luteolysis) and the cycle repeats itself.

The duration of gestation is about 226-240 days, depending on environmental conditions (Guinness *et al*., 1971; Guinness *et al*., 1978; Mattiello and Mazzarone, 2010). The hinds usually give birth to a single calf, rarely to twins (less than 1%), (Sadleir, 1987). The mean birth weight of calves is 8-9 kg (Boitani *et al*., 2003).

In cervids, for most of the gestation, plasmatic concentrations of progesterone are similar to the highest ones reached during the cycle. The mainly source of progesterone during gestation in red deer, seems to be the corpus luteum (Thimonier and Sempere, 1989). Progesterone levels decrease gradually starting from 4 weeks before parturition, reaching basal levels (less than 0.5 ng/ml) at the beginning of lactation (Kelly *et al*., 1982; Adam *et al*., 1985). Probably of foetus-placental origin, oestrogens increase during the whole gestation, reaching higher levels just before parturition (Kelly *et al*., 1982). LH levels in pregnant and non-pregnant females are comparable. Prolactin levels seems to be more affected by photoperiod than by physiological status, decreasing in winter, and increasing in Spring-Summer, both in pregnant and non-pregnant females (Kelly *et al*., 1982).

2.3.5 Assessing reproductive success

In wildlife management, assessing reproductive success is essential: a decrease of fecundity could provide the first indication of a chronic stressor (e.g. an enzootic disease) acting on the population (Lasley and Kirkpatrick, 1991).

The pregnancy status of wild animals has typically been inferred from visual, clinical and laboratory methods (Table 9).

However these approaches have some limitations: for example, direct observation might be difficult in secretive or gregarious species and differences between conception rates and birth rates will not be detected by this approach. Other prenatal techniques (blood sampling, echography, palpation, vaginal histology) require restraint and manipulation of the animal and this may be expensive and stressful in wild deer (Borjesson *et al*., 1996).

Method	Type	Advantages	Disadvantages
Direct observation of recruitment (Solberg and Saether, 1999; Bonenfant *et al*., 2005)	Visual	Quite simple in non-secretive species	Difficult in secretive or gregarious species
			Differences between conception rates and birth rates will not be detected
Palpation (Greer and Hawkins, 1967)	Clinical	Simple, when the animal is well restrained	The animal must be captured and restrained
			Possible iatrogenic embryonic mortality
Ultrasound (Willard *et al*., 1996; Canon *et al*., 1997)	Clinical	Useful and simple in captive animals	The animal must be captured and restrained
			Difficult in the field (equipment, field condition)
Vaginal histology (Matschke, 1977)	Clinical		The animal must be captured and restrained
Determination of serum hormone levels (Loskutoff *et al*., 1983)	Laboratory		The animal must be captured and restrained for sampling
Determination of urine hormone levels (Kirkpatrick *et al*., 1990)	Laboratory	It could be used in uncaptured animals (e.g. using urine-soaked snow)	
Fecal-based radio immunoassay (Deasulniers *et al*., 1989; Messier *et al*., 1990)	Laboratory	The faeces can be sampled after emission, without capturing the animals	It requires special laboratory equipment and the use of dangerous radioisotopes
Fecal-based enzyme immunoassay (Borjesson *et al*., 1996)	Laboratory	The faeces can be sampled after emission, without capturing the animals	
Hair concentrations of progesterone and oestradi (Liu *et al*., 1988; Gleixner and Meyer, 1997)	Laboratory	Progesterone and oestradiol concentration in human hair are correlated with levels measured in serum (Yang *et al.,* 1998)	No reference levels are available for wildlife
		Easy sampling	

Table 9: Methods to assess reproductive status in wildlife.

2.4 Wildlife and environmental chemicals contaminants

2.4.1 Biomonitoring

Biomonitoring of chemical environmental contamination is a very important tool in public health management (Nagheeb Rashed and Soltan, 2005). The content of heavy metals in blood, urine, milk or other biological matrices from humans or animals is indicative of the total load in the organism (Merian, 1991).

Hair could be an interesting substratum for these investigations: the Global Environmental Monitoring System (GEMS) of the United Nations Environmental Program chose human hair as biological matrix (EPA, 2001). Moreover, a number of studies used human hair for microelement detection in ecological, hygienic and clinical investigations (Nowak, 1998; Nowak and Chmieinicka, 2000; Diazbarriga *et al*., 1993; Shah *et al*., 2011).

Due to the exposure of animals to the soil contamination *via* food, animal hair is a better indicator of environmental pollution than the human one; moreover, the accumulation of the substances in the hair occurs as a result of some months of exposure and it represents a long lasting indicator of exposure itself (Merian, 1991; Ray *et al*., 1997).

Wild animals can be used as bioindicators to provide an early warning of potential adverse, contaminant-related effects on organisms or population themselves, on organisms or populations that prey upon them, and as sentinels for exposure and effects on humans (Fox, 2001; Baos et al *et al*., 2006). Furthermore, increasing amounts of meat from wild ungulates are presently available for human consumption (Ramanzin *et al*., 2010), and this may represent a source of toxic,

although the health risk for meat consumers has been considered negligible (Vatheristo *et al*., 2003; Lazarus *et al*., 2008), even in highly polluted areas (Pokorny and Ribarić-Lasnik, 2002).

Several studies on environmental contamination were performed using hair from wild animals: levels of heavy metals were determined in shot moose (*Alces alces*), reindeer (*Rangifer tarandus*), brown bear (*Ursus arctos*), wild boar (*Sus scrofa*) and squirrel (*Sciurus vulgaris*) in Karelia (Medvedev, 1999), in opossum (*Didelphis virginiana*) in Costa Rica (Burger *et al.*, 1994) and in wild boar in Central Italy (Amici *et al*., 2012); mercury concentration in hair was determined in river otter (*Lutra canadensis*) in Georgia (Halbrook *et al*., 1994); environmental contaminants were quantified in ocelots (*Felis pardalis*) from Rio Grande Valley, Texas (Mora *et al*., 2000).

2.4.2 Arsenic (As)

Arsenic is a metalloid element which naturally occurs in organic and inorganic states in soil, air and water (Huang *et al*., 2004; Duker *et al.*, 2005). Organic arsenicals are generally considered nontoxic (Gochfeld, 1995), whereas inorganic forms are toxic. Inorganic arsenic exists mainly in trivalent (As^{3+}) and pentavalent (As^{5+}) forms, where trivalent compounds are more toxic than pentavalent ones (Cervantes *et al*., 1994; Smedley *et al.*, 1996; Duker *et al.*, 2005). Both trivalent and pentavalent arsenicals are soluble over a wide pH range (Bell, 1998) and are regularly found in surface and groundwater (Feng *et al*., 2001). High arsenic concentrations are found in granite and in many minerals including copper, lead, zinc, silver and gold (NAS, 1977).

Arsenic naturally accumulates as both organic and inorganic form in soil, surface and groundwater (Attrep and Anirudhan, 1977), and seawater (Penrose *et al*., 1977). The primary source of arsenic in soil

is the parent rock (Smedley and Kinniburgh, 2002); additionally, volcanoes are a major natural source of arsenic released into the environment (Nriagu and Pacyna, 1988) that can generate high arsenic concentrations in natural waters (Smedley and Kinniburgh, 2002).

Arsenic accumulates within the soil, water and air, where it is subsequently taken up and processed by microbes, plants and animals. Soluble arsenic taken up by plants rapidly accumulates in the food chain. The major sources of exposure for humans and other terrestrial mammals are food and water (Bernstam and Nriagu, 2000).

Once ingested, arsenic that is not eliminated from the body may accumulate in the muscles, skin, hair and nails (Ishinishi *et al*., 1986; Kitchin, 2001). Both organic and inorganic arsenic are present in food, whereas water primarily contains inorganic forms. As an element arsenic is poorly absorbed, and it is predominantly eliminated from the body without biotransformations (Duker *et al.*, 2005). Inorganic arsenic is absorbed through the gastrointestinal tract and it is eliminated via renal function (Hindmarsh and McCurdy, 1986); however, a small amount is biotransformed via methylation and reduction in the liver (Winski and Carter, 1995; Bernstam and Nriagu, 2000): methylation of arsenic may, in some cases, increase arsenic toxicity in humans and rodents (Petrick *et al*., 2000; Petrick *et al*., 2001; Styblo *et al*., 2000; Del Razo *et al*., 2001).

Arsenic inhibits more than 200 enzymes (Abernathy *et al*., 1999) and it has been implicated in multisystemic health effects via interference with enzymatic function and transcriptional regulation (NRC, 1995). A number of inhibitory effects have been described, as the ones affecting mitochondrial respiration (Klaassen, 1996; Abernathy *et al*., 1999) and synthesis of adenosine triphosphate (ATP) (Winship, 1984). Further effects of arsenic are: activation of the estrogen receptor,

inhibition of angiogenesis and tubuline polymerization, induction of heat-shock proteins, and oxidation of glutathione (Bernstam and Nriagu, 2000).

Chronic exposure to arsenic may determine immunosuppression in many mammals, affecting lymphocyte, monocyte and macrophage activity (Blackely *et al*., 1980; Gosenbatt *et al*., 1994; Lantz *et al*., 1994; Yang and Frenkel, 2002; Wu *et al*., 2003; Duker *et al.*, 2005; Sakurai *et al*., 2006; Lage *et al.*, 2006).

Arsenic may have acute toxicity for organs involved with absorption, accumulation or excretion, as skin, circulatory system, gastrointestinal tract, liver and kidney (Duker *et al.*, 2005; Madden and Fowler, 2000). Some Authors evidenced a role of arsenic in the development of cystine calculosis (Golabek *et al*., 2004). Furthermore, long-term exposure to inorganic arsenic in humans is associated with certain forms of cancer of the skin, lung, colon, bladder, liver and breast (Abernathy *et al*., 1999; Smith *et al.*, 2000; Abernathy *et al.*, 2003; Huang *et al*., 2004; Duker *et al.*, 2005).

3 The use of hair to assess the status in wild red deer (*Cervus elaphus*, Linnaeus, 1758) populations

3.1 Introduction

3.1.1 Why hair?

In the last decades, hair have been used in human medicine to extract DNA (Woodruff, 1993; Morin *et al*., 1994), to reveal trace metals (Eads and Lambdin, 1973; Foo *et al*., 1993), to quantify chronic stress indicators and prenatal exposure to drugs in hospitalized neonates (Yamada *et al*., 2007), to find out doping agents in athletes controls (Raul *et al*., 2004; Cirimele *et al*., 2000; Bowers and Segura, 1996; Kintz, 2003), to assess drug-related fatalities, and criminal responsibilities (Wenning, 2000).

For some years now, the use of this matrix has been introduced also in animal sciences to investigate HPA axis activity, providing reliable information on chronic stress with no or very little disturbance for the animals (Koren *et al*., 2002; Davenport *et al*., 2006; Accorsi *et al*., 2008; Comin *et al*., 2011; Comin *et al*., 2012 a; Comin *et al*., 2012 b).

Hair is a safe, readily available, and easy to store and transport matrix, and hair sampling does not involve pain or infection risk for the animals (Koren *et al*., 2002). Furthermore, hair assay provides a long-term endocrine profile, without the interference of circadian biorhythms and acute stress, including that caused by handling the animals during the sampling (Prandi *et al*., 2010). Thus, this matrix could be useful to assess chronic stress and other hormonal substrates of social trends, but awaits validation for different species (Accorsi *et al*., 2008). Furthermore, hair can be an important indicator of

accumulation of environmental pollutants in ecological, clinical and hygienic studies.

In particular, in wildlife ecology, its use could be very interesting, reducing the necessity to capture and handle the animals for sampling (e.g. Belant *et al*. (2007) describe the use of hair snares in white-tailed deer to collect hair samples without animals restraint), and allowing to test a great number of individuals in a population, with low costs and little disturbance.

3.1.2 Mechanism of incorporation of substances in the hair

To now, the mechanism that permits the incorporation of different substances in the hair and the way these substances actually move along the hair shaft are not well understood (Kirschbaum *et al*., 2009). Different models have been proposed to explain these phenomena.

The first model suggests that incorporation occurs simply for passive diffusion from the blood stream into the growing cells at the base of the hair follicle during growing phase (anagen phase): this incorporation would end with the keratinization and dehydration of hair cells (Cone, 1996). However, this model is now considered over-simplified: so another model was proposed, assuming a deposition of substances from deep dermal layers during the growing, from sebaceous and apocrine glands wetting growing hair, as well as from the external environment (Henderson, 1993). This model is now usually accepted from the most Authors (Pragst *et al*., 1998; Kintz, 2004).

It is important to note that diffusion is amplified by high lipids solubility and by a low proteic binding: for that reason, steroids measured in the hair are related with the free part of steroids in the blood (Prandi *et al*., 2010).

3.2 Study area

Sondrio Province (LAT. 46.01233-46.63044; LONG. 9.24462-10.63960) (Figure 10) is located in the North Lombardy Region, in central Italian Alps. It includes the Adda River Valley (Valtellina) and Mera River Valley (Valchiavenna), besides the Livigno and Lei Valleys, which are afferent to the Reno hydrographical basin. It confines with Swiss at northern and western sides, with Bolzano and Trento Provinces at eastern side, and with Brescia, Bergamo, Lecco and Como Provinces at southern side. The total surface is about 3,197 km^2; the maximum length East-West is 119 km, the maximum width North-South is 66 km.

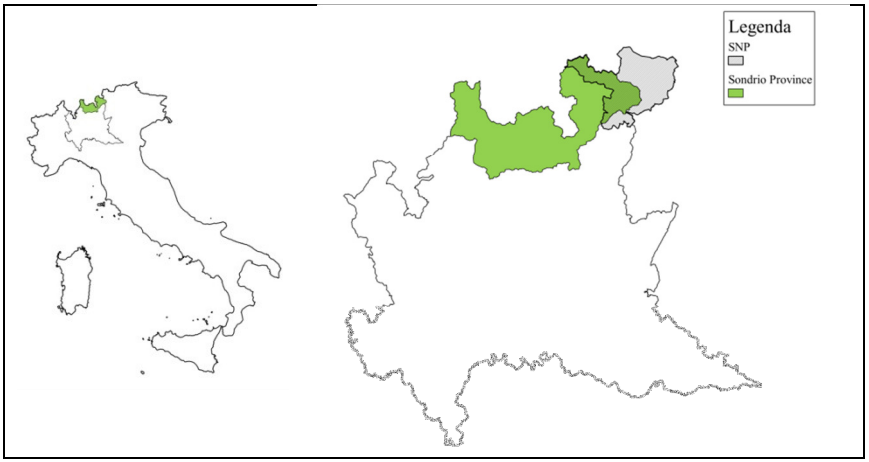

Figure 10: Position of Sondrio Province with respect to Lombardy and Italy.

About 70% of the territory is situated above 1,500 m a.s.l.; the highest altitude reached is 4,021 m a.s.l. at the top of Pizzo Bernina. Retic Alps occupy the right hydrographical side of the Adda Valley, the left side upstream the Aprica Pass and the left hydrographical side of Chiavenna and San Giacomo Valleys. From the Como Lake and the

Aprica Pass, the southern side is constituted by Orobic Alps, less high than the Retic ones, with the highest peak (Pizzo Coca) at 3,052 m a.s.l.. The right sides of Chiavenna and San Giacomo Valleys are part of the Lepontine Alps, with the highest peak (Pizzo Tambò) at 3,279 m a.s.l.

From a geological point of view, the territory is subdivided into two different parts from Insubric Line (West-East direction): South-alpine and North-alpine. The first is constituted of sedimentary formations resting on ancient metamorphic rocks; the latest is extremely complicated and it is constituted of two systems of overlapping layers.

Climate is characterized by annual average temperatures varying from the isotherm of 12°C (lake, moraine area and lowest altitudes), to the one of 2.5°C (alpine area, from 1,700 to 2,400 m a.s.l.), up to the one of 0°C above 2,900 m a.s.l. Annual thermal excursion (difference between the average temperature in the wormer month and the average temperature in the colder one) is influenced primarily by geomorphology and varies from 21.6°C (low altitudes) to 14°C (alpine environment). Two rainfall regimes are described: the alpine (or continental) one, with one summer peak and the sublitoral-alpine one, with two peaks (in Spring and Autumn). Substantially, we can identify three types of climate: i) sub-alpine (cold season enduring 4 months); ii) alpine (above tree line, with harsh winters enduring 6 months); iii) glacial (with temperatures under 0°C, almost exclusively snowy precipitations and almost absent vegetation) (Ferloni, 2012).

The main tree and herbal species characterizing the different altitudinal levels are summarized in Table 10.

Level	Altitude range (m a.s.l.)	Tree species	Herbal species
Sub-mountain	500-1,000	*Fagus sylvatica* *Abies alba* *Larix decidua* *Picea abies* *Sorbus aucuparia* *Cytisus laburnum* *Calluna vulgaris*	*Trisetum flavescens* *Trifolium montanum* *Ranunculus montanus* *Campanula barbata* *Trolius europaeus*
Mountain	1,000-1,400	*Picea abies* *Larix decidua* *Vaccinium* spp. *Rhododendrum ferrugineum* *Rubus idaeus*	*Melampyrum sylvaticum* *Campanula barbata* *Veronica officinalis*
Sub-alpine	1,400-1,800	*Larix decidua* *Pinus cembra* *Pinus montana var. mughus* *Picea abies* *Alnus viridis* *Juniperus communis var. nana*	*Festuca ovina capillata* *Nardus stricta* *Trifolium montanum*
Lower alpine	1,800-2,400	*Rhododendrum ferrugineum* *Vaccinium* spp. *Pinus montana var. mughus* *Alnus viridis*	*Nardus stricta* *Carex ferruginea* *Salix pentantra* *Salix purpurea*
Alpine	2,400-2,700	---	*Carex curvula* *Carex firma* *Carex elyna*
Nival	> 2,700	---	*Carex curvula* *Carex firma* *Carex elyna* *Saxsifraga panicolata* *Saxifraga aizoon*

Table 10: summary of main vegetal species in different altitudinal levels (Ferloni, 2012).

3.3 Red deer distribution, presence, density and management in Sondrio Province and in Stelvio National Park (SNP)

Currently, red deer shows a continuous distribution in the Alps: this is the result of a series of re-introductions actuated after the World War II, when the species was almost completely exterminated in Italy (with the exception of some small and isolated groups in central-eastern Alps, Bosco della Mesola, and Sardinia (*Cervus elaphus corsicanus*) (Mustoni *et al*., 2002; Mattiello and Mazzarone, 2010)), and the natural expansion from neighbouring European countries (Pedrotti et al., 2001). In the last years, in Italy, red deer number reaches about 63,000 individuals, distributed mainly in the Alps (78%) (Carnevali *et al*., 2009).

The species is stably present in most of the territory of Sondrio Province, but it tends to avoid areas with high snow cover during the winter and highly anthropized areas. In the Retic Alps the distribution is homogeneous, while it is discontinuous and just at low altitudes in the Orobic mountainside. In the SNP the species is well distributed and it avoids just some peaks and the glaciers (Ferloni, 2012).

Data referred to the censuses and culling plans in the districts involved in this thesis are reported in Table 11.

On average, density ranges between 1 and 4 individuals/km^2, although in the CA Alpine District Alta Valle (CA-AV) and in the SNP it reaches generally higher densities, up to 40 individuals/km^2 in some winter areas in SNP (Pedrotti, *in verbis*).

CA/districts	Abbreviation	Hair samples (n)	Total suitable area (km^2)	Counting area (km^2)	Pre-reproductive census (n)	Pre-reproductive density (individuals/km^2)	Permitted heads (n)	Hunting rate (hunted heads/permitted heads)	Culled individuals (n)	Culling density (individuals/km^2)	Hunters (n)	Residents (n)
CA-MO		**30**	**449.09**									
Val Masino	MO3	30	163.81	131	256	2	62	24.2	63	0.38	95	7853
CA-SO		**17**	**674.22**									
Val Madre	SO8	3	50.489	12.47	14	1.1	0	0				2131
Arcoglio	SO1	9	102.67	43.66	327	7.5	100	30.6	79	0.77	122	7440
Val di Togno	SO3	1	68.257	65.9	104	1.6	30	28.8	20	0.29	50	6998
Val Fontana	SO4	4	70.71	63.66	179	2.8	36	20.1	26	0.37	57	4840
CA-AV		**40**	**352.52**									
San Colombano	AV2	9	72.99	50.22	91	1.8	36	39.6	22	0.30	51	3481
Storile	AV1	17	39.909	24.23	117	4.8	50	42.7	34	0.85	30	4301
Val Viola	AV3	14	91.635	29.71	101	3.4	44	43.6	27	0.29	48	4033
SNP		**89**	**316.7**	**316.7**	**1770**	**5.6**						**6819**
Valfurva		89	90	90	1100	12.2	100		98	1.09	98	

Table 11: data referred to the Red Deer censuses and culling in 2011 in the different districts involved in this thesis (Ferloni, 2012). The Hunter (Source: Ferloni, *in verbis*) and Resident (Source: ISTAT, 2009) numbers are indicative of anthropic disturbance.

In some areas, the elevated number of individuals causes damage to the agriculture and forestry or determines an increase of road accidents, so an accurate culling management is required.

In Italy most culling is open to anyone, because game is not considered the property of the landowner (law n° 157/1992). In the Lombardy region, the territory of provinces is divided into General Hunting Districts (ATC: Ambito Territoriale di Caccia) and CA Alpine Districts (CA: Comprensorio Alpino), further subdivided in ungulates hunting districts (Apollonio *et al*., 2010).

Regarding red deer hunting management, Sondrio Province is subdivided in 5 CA districts, according to the national law n° 157/1992 and to the regional law n° 26/1993: CA-Chiavenna (CA-CH), CA-Morbegno (CA-MO), CA-Sondrio (CA-SO), CA-Tirano (CA-TI) and CA-Alta Valle (CA-AV); the CA districts, further subdivided in ungulate hunting districts (according to the regional law n° 16/2003), are shown in Figure 11.

On the Retic side of the Province a territory of about 78 km^2 is occupied by two Game Hunting Farms (Azienda Faunistico Venatoria=AFV Valbelviso-Barbellino and AFV Val Bondone-Val Malgina), in which culling is open just to paying members.

The eastern side of the Province is occupied by a sector of the Stelvio National Park (SNP) and it hosts a numerically important red deer population.

According to law n° 394/1992, general public cannot cull ungulates in protected areas, but rangers or hunters with special training and authorisation under rangers surveillance can control them. Before 2011, the only culling programme for red deer in a protected area was

performed starting from 2000 in the South Tyrol sector of SNP: it had the purpose to reduce the red deer population number in order to reduce browsing impact on spruce (*Picea abies*) forest regeneration (Apollonio *et al*., 2010).

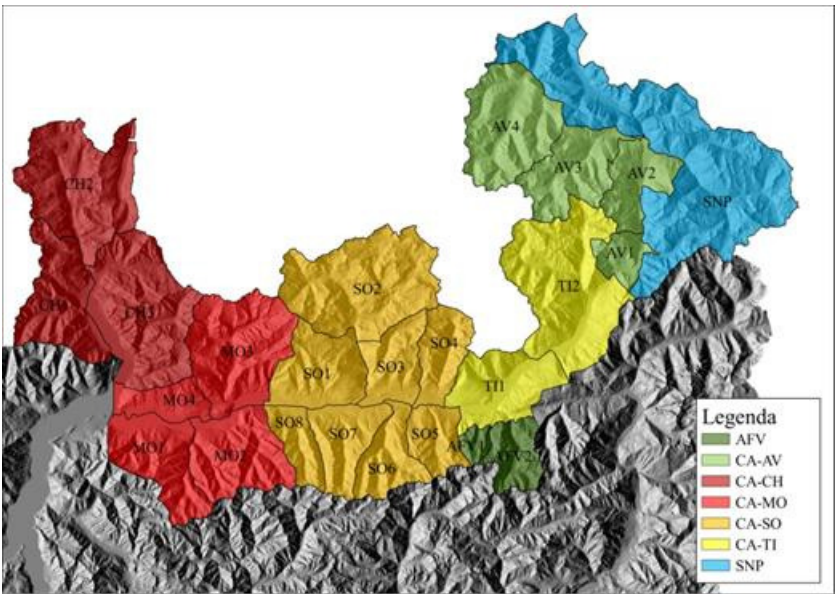

Figure 11: Orography of Sondrio Province with the location of the CA districts (further subdivided into ungulate hunting districts), of the Game Hunting Farms (AFV) and of the Stelvio National Park (SNP).

In some areas of the Sondrio sector of the SNP, the density of red deer in some winter areas reaches 40 heads/km^2 (density on actually occupied area), probably due to the effect of high culling regimes in the neighbouring areas. In order to reduce the density of red deer inside the Park and to control excessive red deer impact on agriculture, forestry and road killings, in 2011 a culling programme was started in Valfurva, in the Sondrio sector of the SNP.

3.4 Hair cortisol assessment in Red Deer

3.4.1 Introduction

Cortisol has long been considered a reliable physiological measure of the allostatic load both in domestic and in wild mammals (see chapter 2.1.1). Chronic high levels of cortisol and other glucocorticoids may lead to a pathological syndrome characterized by metabolic modifications (as increase of gluconeogenesis, lipidic and proteinic catabolism) and depression of reproductive and immune activity, and has direct activity on the central nervous system (see paragraph 2.1.3.2) (Moberg, 2000; Charmandari *et al.*, 2005; Macbeth *et al*., 2010).

Assessing the hypothalamus-pituitary-adrenal (HPA) axis activity could be an important tool in wildlife management (McLaren *et al.*, 2007). A chronic stimulation of HPA axis leads to an increase of energetic costs for the animal and it can be especially significant in wildlife living in poor environmental conditions or during particular life stages (Reeder and Kramer, 2005).

Measuring cortisol in blood, urine, faeces and saliva is strongly affected by diurnal and seasonal variation, sampling method, diet, physiological status, environmental conditions (Von der Ohe and Servheen, 2002; Owen et a *et al*., 2005; Constable *et al*., 2006; Keay *et al*., 2006). Furthermore, cortisol in these matrices indicates short term HPA axis activation, not assessing HPA axis activity occurring over weeks to months without repeated sampling of individuals (Owen *et al*., 2005; Keay *et al*., 2006).

Hair Cortisol Concentration (HCC) was found positively correlate to salivary cortisol concentration in rhesus macaques (Davenport *et al*., 2006), and to faecal cortisol concentration in dogs and cats (Accorsi *et*

al., 2008). HCC in an intact hair shaft should represent an integrated measure of systemic HPA axis activity during the active growth phase of the hair being evaluated (Macbeth *et al*., 2010), due to the process of cortisol incorporation in hair (see paragraph 3.1.2).

Long term cortisol concentrations have been investigated in hair in a number of domestic or laboratory species in the last years (Koren *et al*., 2002; Davenport *et al*., 2006; Accorsi *et al*., 2008; Bennet and Hayssen, 2010; Comin *et al*., 2011; Comin *et al*., 2012 a; Comin *et al*., 2012 b; Montillo *et al*., 2012) (see paragraph 3.1.1). To our knowledge, on the contrary, scarce information are available about HCC in free-ranging large mammals: just one study was carried out on grizzly bear (*Ursus arctos*) by Macbeth *et al.* (2010), and this is the first study concerning HCC in free-ranging wild red deer.

Nevertheless, the possibility to investigate on long-term HPA axis activity without causing disturbance to the animals and collecting a large numbers of samples could be an interesting tool in wildlife management. Hair snares are already used in genetic research on free-ranging wild mammals (Woods *et al*., 1999; Mowat and Paetkau, 2002; Dobey *et al*., 2004; Belant *et al.*, 2007) and could be used also to obtain hair samples to assess the status of populations, with regard to long-term HPA axis activity and allostatic load.

The aims of this study are: i) to develop a dependable method to measure HCC in non-invasively collected sample from wild red deer; ii) to examine HCC in relation to biometric measures, age and sex classes in this species.

3.4.2 Materials and methods

3.4.2.1 Samples collection

The subjects for this study were 174 red deer: 85 samples were from the CAs (CA Morbegno=CA-MO, CA Sondrio=CA-SO and CA Alta Valle=CA-AV), and 89 samples were from SNP. The number of available samples (with relative percentages) for sex and age classes for each CA, for the SNP, and in the total is shown in Table 12 and Table 13.

CA	Males		Females		Total
	n	%	n	%	
CA-MO	11	39.29	17	60.71	28
CA-SO	8	47.06	9	52.94	17
CA-AV	23	57.5	17	42.5	40
SNP	34	38.2	55	61.8	89
Total	76	43.68	98	56.32	174

Table 12: number and relative percentages of available samples for each sex in each CA and for the SNP.

CA	Calves		Yearlings		Adults		Total
	n	%	n	%	n	%	
CAC_MO	8	28.57	8	28.57	12	42.86	28
CAC_SO	9	52.94	4	23.53	4	23.53	17
CAC_AV	10	25	5	12.5	25	62.5	40
PNS	30	33.71	11	12.36	48	53.93	89
Total	57	32.75	28	16.09	89	51.15	174

Table 13: number and relative percentages of available samples for each age class in each CA (Calves=0 year; Yearlings=1 year; Adults≥2 years).

Hair samples were torn off from the wither of red deer culled during the 2011 hunting season in CAs (September-December) and 2012 biological control in SNP (January and February). Each animal was identified by a numerical code; sex, age and biometrics measures (body length, foot length, withers height, jaw length, carcass weight) were recorded in a dataset with data relative to culling site, date and

time. Hair samples were dried if necessary and stored in paper envelopes at room temperature until analysis.

3.4.2.2 Hair cortisol assay

In laboratory, hair strands were placed in polypropylene tubes with the addition of 5 ml isopropanol, and then the samples were gently mixed on a rotator at room temperature for 3 minutes per wash: this relatively short wash time is necessary to remove steroids present in sweat/sebum from the external surface of the hair, minimizing the risk of cortisol extraction from the interior of the hair shaft (Davenport *et al*., 2006). The hair was then allowed to dry for approximately 5 days in a clean protected hood (Paulsen *et al*., 2001).

Hair cortisol was extracted according to the method described by Koren et al. (Koren *et al*., 2002), with some modification (Comin *et al*., 2012 b). Approximately 60 mg of trimmed hair were placed in a glass vial along with 3 ml of methanol and incubated at 37°C for 18 hours (Figure 12). Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.6 ml of phosphate-buffered saline (PBS) 0.05 M, pH 7.5.

Hair cortisol was measured using a solid-phase microtitre RIA procedure (Tamanini *et al*., 1983; Comin *et al*., 2011). In brief, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with anti-rabbit γ-globulin serum raised in a goat, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.4 and incubated overnight at 4°C with 200 μL of the anti-cortisol serum diluted 1:12000 (Figure 13). The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). Cross-reactivities of this antibody with steroids are: cortisol 100%,

corticosterone 1.8% and aldosterone<0.02%. After washing the plate with RIA buffer, standards (5-300 pg/well), a quality control extract, the test extracts and tracer (Hydrocortisone (Cortisol, [1,2,6,7-3H (N)]-), Perkin-Elmer Life Sciences, Boston, MA, USA) were added and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). Intra-assay and inter-assay coefficients of variation were 3.6% and 9.8%, respectively. The assay sensitivity, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 1.23 pg/well.

Figure 12: trimmed hair are added with 3 ml of methanol and incubated at 37°C for 18 hours (photo: C. Caslini).

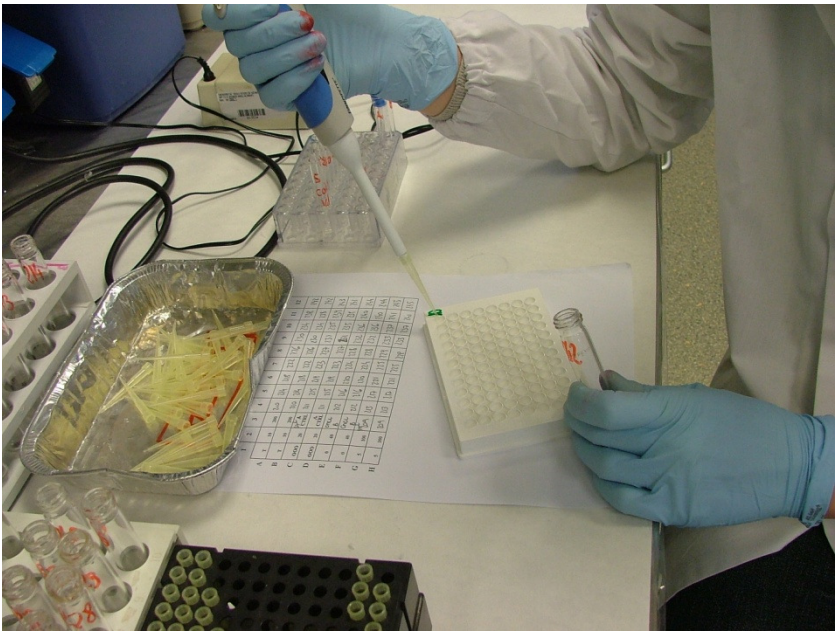

Figure 13: preparing the 96-well microtitre plate (photo: C. Caslini).

3.4.2.3 Statistical analysis

The concentration data, expressed in pg/mg, were stored using MS Excel 2010. Statistical analysis were performed with SPSS 13.0 software. Graphs were performed with SPSS 13.0 software or MS Excel 2010.

Differences among sexes and age classes were tested by Mann-Whithney U-test.

A Generalized Linear Model (GzLM) was used to analyse variation in HCC including the effects of sex, age class, sex*age class, data and culling area.

A Generalized Linear Model (GzLM) was used to analyse variation in Carcass Weight (CW) including the effects of sex, age class, sex*age class, and culling area.

HCC classes were created on the basis of HCC values, in order to allow a better graphical representation of data.

A Spearman correlation test was performed for HCC, biometric measures (body length (BL), foot length (FL), withers height (WH), jaw length (JL), carcass weight (CW)), and Kidney Fat Index (KFI). All correlations between HCC and biometrics measures were tested using a partial correlation, corrected for age, in order to exclude age effect from the results.

A probability *P* value of less than 0.05 was considered significant.

3.4.3 Results

No differences between sexes were evidenced for HCC (Mann-Whitney U=3625.0; $P>0.05$). In consequence of that result, in subsequent analysis, males and females were considered together.

The distribution of HCC values for each age class, in each area and in the total sample is shown in Table 14.

Statistical differences in HCC among age classes were detected with repeated Mann-Whitney U-test: calves *versus* yearlings (Mann-Whitney U=400.0; $P<0.001$); calves *versus* adults (Mann-Whitney U=1968.0; $P<0.05$). Difference is very close to the statistical significance between yearlings and adults (Mann-Whitney U=940.0; $P=0.051$).

Calves						
Culling area	**n**	**minimum**	**25th percentile**	**median**	**75th percentile**	**maximum**
CA-MO	8	3.97	4.49	4.65	5.8	7.85
CA-SO	9	4.15	4.64	5.78	6.81	6.95
CA-AV	10	3.72	4.76	5.6	6.97	12.53
SNP	30	3.84	5.56	6.48	9.05	29.19
TOTAL	57	3.72	4.77	5.92	7.72	29.19
Yearlings						
Culling area	**n**	**minimum**	**25th percentile**	**median**	**75th percentile**	**maximum**
CA-MO	8	2.09	3.04	3.96	4.25	6.12
CA-SO	4	4.31	4.4	5.48	6.53	6.6
CA-AV	5	5.13	5.24	5.55	6.34	6.64
SNP	11	3.07	3.6	4.66	5.77	7.29
TOTAL	28	2.09	3.92	4.65	5.82	7.29
Adults						
Culling area	**n**	**minimum**	**25th percentile**	**median**	**75th percentile**	**maximum**
CA-MO	12	2.49	2.95	4.59	4.95	6.04
CA-SO	4	5.31	5.36	6.13	6.81	6.83
CA-AV	25	2.72	4.94	6.44	8.49	18.66
SNP	48	2.9	4.33	4.9	7.15	43.18
TOTAL	89	2.49	4.36	5.07	7.07	43.18

Table 14: HCC (pg/mg) minimum, 25th percentile, median, 75th percentile, and maximum in each age class, for each culling area and in the total sample.

With GzLM, no significant differences in HCC among age classes were detected (calves: 6.17±0.66 pg/mg; yearlings: 4.47±0.83 pg/mg; adults: 5.15±0.74; last square mean±SE; $P>0.05$), although HCC difference between calves and yearling is very close to statistical significance ($P=0.059$).

Given the limited number of yearlings and considering that no differences in HCC were evidenced between yearlings and adults, in subsequent analysis, these two age classes were considered together for subsequent analysis.

Applying GzLM, HCC differs significantly (P=0.01) between CA-MO (2.87±1.56; least square mean±SE) and CA-AV (6.07±0.89; least square mean±SE), and the HCC is almost different (P=0.06) between CA-MO and SNP (7.45±1.01; last square mean±SE), but not between other areas (Figure 14).

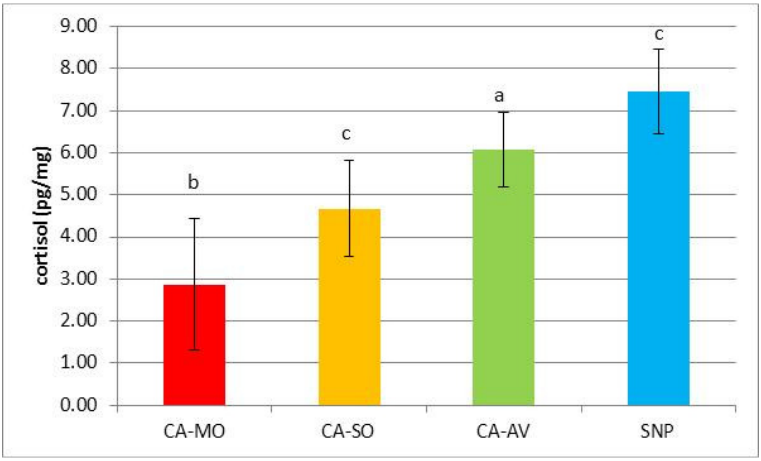

Figure 14: Comparison of HCC in the 4 culling areas. Least square means are represented by bars and standard errors are represented by error bars. Significant difference ($p \leq 0.05$) in HCC was detected between "a" and "b", but no significant differences were detected between "a" and "c" and between "b" and "c".

Applying GzLM, considering sex, age class, sex*age class, and culling area effects, CW differs significantly (P<0.001) between SNP (46.74±1.49; least square mean±SE) and respectively CA-MO (62.71±4.01; least square mean±SE), CA-SO (61.73±2.9; least square mean±SE) and CA-AV (62.07±2.04; least square mean±SE).

HCC obtained were grouped in HCC classes. The percentages of calves and yearling+adults falling in each HCC class are showed respectively in Figure 15 and Figure 16.

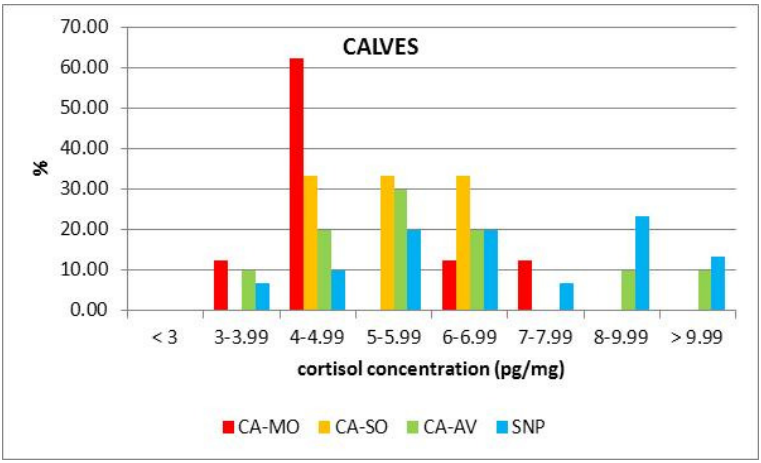

Figure 15: Percentage of calves from each culling area in each HCC class.

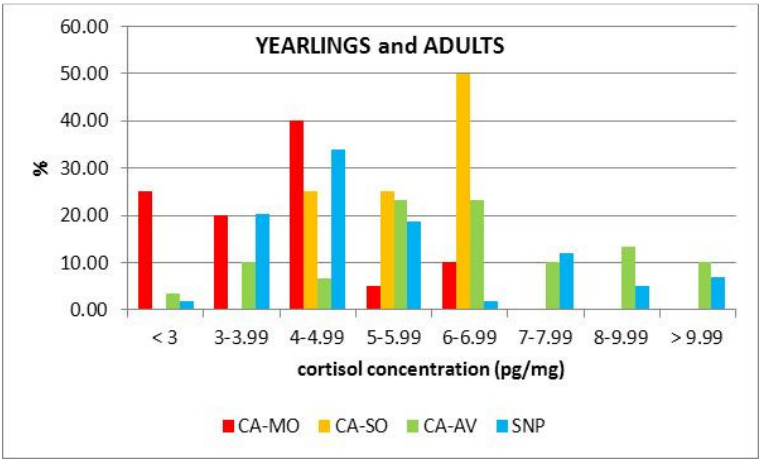

Figure 16: Percentage of yearlings+adults from each area in each HCC class.

Significant negative correlations were detected between HCC and withers height (correlation coefficient=-0.179; $P<0.05$), and HCC and carcass weight (correlation coefficient=-0.161; $P<0.05$). Partial

correlations, corrected for age (HCC-withers height: correlation=-0.048; $P>0.05$; HCC-carcass weight: correlation=-0.052; $P>0.05$) were not significant. No significant correlations were detected between HCC and other biometric measures tested ($P>0.05$).

3.4.4 Discussion

The aim of this study were: i) to develop a dependable method to measure cortisol concentration in hair from wild red deer; ii) to examine HCC in relation to culling areas, biometric measures, age and sex classes in this species. Because to our knowledge this is the first study carried out on HCC in free-ranging wild red deer, we were not able to compare our results with others related to the same species.

The absence of difference among sexes allowed us to consider males and females together in subsequent analysis. The higher HCC we found in calves compared to other age classes is similar to the one found by Maiero *et al.* (2005) in cattle. The weaning period may explain this high concentration: in red deer, when the mother is once again pregnant, the weaning occurs at the calf age of 5-7 months; if the mother is not pregnant, it occurs during the summer (Mattiello and Mazzarone, 2010). Our samples were from calves 7-8 months old, and so supposedly weaned or close to the weaning: this phase could generate a stress in calves (Griffin *et al.*, 1988; Pollard *et al.*, 1992; Zavy *et al.*, 1992; Pollard *et al.*, 1998), justifying the higher HCC we found in calves than in other age classes.

From a wildlife management point of view, one of the most important differences among culling areas is the red deer density, in particular during the winter (see paragraph 3.3): in the whole study area it ranges between 1 and 4 individuals/km^2, although in the CA-AV and in the SNP it reaches generally higher densities, up to 40 individuals/km^2 in some winter areas in SNP (Pedrotti, *in verbis*). High density

populations are usually subject to a series of problems like infertility, decrease of body weight (Vincent et al *et al*., 1995; Toïgo *et al*., 2006), behavioural alterations, and increasing of faecal cortisol concentration (Li *et al*., 2007). Our findings reflect this datum: HCC in red deer from CA-MO is significantly different from CA-AV and almost significantly different from SNP, the two areas with higher density and in which environmental conditions are harder. The difference between CA-AV/SNP and other culling areas is even more noticeable observing Figure 15 and Figure 16: only in CA-AV and SNP some individuals reach HCC greater than 8.0 pg/mg, up to 43.18 pg/mg in an individual from SNP. Individuals from CA-MO, in which the lowest HCC in the total sample was detected, are concentrate mainly in the lowest cortisol concentration classes. CA-SO shows the distribution that seems the most homogeneous: all the individuals are indeed concentrated in the three central cortisol concentration classes, with no extremely high and no extremely low values.

The significantly lower CW recorded in SNP compared to that of other culling areas, further supports the hypothesis of a higher allostatic load in the SNP rather than in the other areas, probably due above all to the high density of red deer in the Park. In wild ungulates populations, in fact, body measures can decrease with increasing density (Toïgo *et al*., 2006) and our findings seem to confirm that.

The negative correlations between HCC and withers height and HCC and carcass weight are actually biased by the effect of age class and cannot be considered reliable: in fact, if age effect is introduced for the calculation of partial correlations, results are no longer significant. Therefore, the negative correlation was most probably due the presence of calves (with higher HCC and smaller dimensions than other age classes) in the sample.

Findings concerning differences in HCC and CW between culling areas and the distribution of HCC classes in the different culling areas seem to indicate an increase of allostatic load in SNP and in CA-AV, but we can just suppose the real cause of this increase. Certainly, high population density can increase energetic requests, through the increasing of interaction between individuals, and the decrease in trophic resources availability. Another cause could be the anthropic disturb: SNP and neighbouring areas are very popular tourist destinations, both in winter and in summer; this may represent an important source of disturbance not only in summer, but also and especially in winter, when snow cover is deep and it makes harder for deer to move and to find food, increasing energy expenditure (Jeppesen, 1987; Schmidt, 1993). Furthermore, several domestic herds (mainly dairy cows) reach the high pastures of SNP during the summer and can cause disturbance to wild red deer, especially during milking procedures (Mattiello *et al*., 2002; Mattiello *et al*., 2003). High prevalence of some disease may be another important reason for increasing allostatic load: in SNP red deer population reaches a high prevalence for paratubercolosis (Bianchi, *in verbis*), a chronic disease, caused from *Mycobacterium avium* subspecies *paratuberculosis*, that leads animals to death for dehydration and severe cachexia.

In conclusion, the assessment of cortisol concentration in the hair seems to be an interesting tool for future wild red deer management, although further investigations are required. Such a non-invasive method to assess the populations status could have an important role in free-ranging large mammals research and management, reducing sampling efforts for researches and disturbance and risks for animals. Using the method we proposed, associated to a sample collection by hair snares (Belant *et al.*, 2007), wide spectrum investigations on free-ranging population status could be easily achievable.

3.5 Hair progesterone (P4) concentration assessment in Red Deer

3.5.1 Introduction

In wildlife management, assessing reproductive success is essential: a decrease of fecundity could provide the first indication of a chronic problem (e.g. an enzootic disease) acting on the population (Lasley and Kirkpatrick, 1991), and which may have serious effects on population dynamics.

However, the current methods used to assess reproductive status are often difficult to apply in wild ungulates, because they require restraint and manipulation of the animal or because of particular field conditions (e.g., direct observation might be difficult in secretive or gregarious species) (see paragraph 0).

In this study we investigate possible relation between Hair P4 Concentrations (HP4C) and reproductive status in free-ranging culled red deer females. This represents a preliminary research, aiming at defining the profiles of reproductive hormones in red deer hinds, in order to identify possible indicators and patterns that may be of help for setting up reliable and easy to use methods for pregnancy diagnosis using hair as matrix.

3.5.2 Materials and methods

3.5.2.1 Samples collection

For this study, hair from 54 red deer females culled in SNP were collected: 13 calves (0.5 years), 2 yearlings (1.5 years), 39 adults (≥ 2.5 years). Thirty-three individuals were culled in January and 21 in February, at a distance varying from 16 to 26 days.

Hair was torn off from withers area; after that, it was dried if necessary and stored in paper envelopes at room temperature until analysis.

Each individual was necropsied in order to detect with certainty the presence/absence of the foetus: all the calves and the yearlings resulted not pregnant; out of the 39 adults, 35 were pregnant, and 4 not pregnant.

Calves were excluded from statistical analysis, because of the immaturity of their reproductive apparatus: their HP4C is not necessarily related to their reproductive status.

3.5.2.2 Extraction from hair

Hair extraction was performed using methanol and the progesterone levels were determined by the RIA method. Briefly, the hair strands were washed in 5 ml isopropanol, as suggested by Davenport *et al*. (2006), and the 60 mg of trimmed hair was put in a glass vial and extracted with 3 ml of methanol. The vials were incubated at 37°C for 18 h. Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.3 ml of phosphate-buffered saline (PBS), 0.05 M, pH 7.5.

3.5.2.3 Progesterone assay

Hair progesterone levels were measured using a solid-phase microtitre RIA assay. In brief, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and the plate was incubated overnight at 4°C. The plate was then washed twice with RIA buffer, pH 7.4, and incubated overnight at 4°C with 200 µl of the rabbit anti-11α-OH-progesterone-hemisuccinate-BSA antibody produced in our laboratory diluted

1:8.000. Cross-reactivities of this antibody with other steroids are as follows: 11 β-OH-progesterone, 46%; 17 α-OH-progesterone, 0.4%; 20α-OH-progesterone, 0.04%; testosterone, 0.08%; cortisol, <0.01%; estradiol 17β, <0.01%; estradiol 17α, <0.01%; and estrone, <0.01%. After washing the plate with RIA buffer, standards (5–300 pg/well), a quality control extract, the test extracts and tracer (Progesterone [1,2,6,7-3H (N)]-, Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µl scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). Intra-assay and inter-assay coefficients of variation were 4.06% and 11.47%, respectively. The sensitivity of the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 0.56 pg/well.

3.5.2.4 Statistical analysis

The concentration data, expressed in pg/mg, were stored using MS Excel 2010. Statistical analysis were performed with SPSS 13.0 software. Graphs were performed with SPSS 13.0 software or MS Excel 2010.

Differences in HP4C between pregnant and not pregnant females were tested by Mann-Whitney U-test. Differences in HP4C between individuals culled in January and in February were tested by Mann-Whitney U-test.

A Spearman correlation test was performed for HP4C, biometric measures (body length, foot length, height at withers, jaw length, carcass weight), and Kidney Fat Index (KFI).

A logistic analysis was performed in females older than 2 years (n=39), in order to investigate the dependence of pregnant/not pregnant status from HP4C, Hair Cortisol Concentration (HCC), Kidney Fat Index (KFI), age class, biometric measures (body length, foot length, height at withers, jaw length, carcass weight), and culling month.

P<0.05 was considered to indicate statistically significant differences.

3.5.3 Results

Descriptive statistics of HP4C in pregnant and not-pregnant hinds are shown in Table 15, while in Figure 17 HP4C for each individual (pregnant, not-pregnant and, just for completeness, calves) is shown.

	n	Minimum	Maximum	Mean	Standard deviation
Not pregnant	6	7.69	26.63	15.51	6.73
Pregnant	35	8.93	84.13	32.12	19.35
Calves	13	13.01	41.04	23.46	8.15

Table 15: descriptive statistics of HP4C (pg/mg) in pregnant and not-pregnant red deer hinds and in calves.

All not-pregnant hinds showed HP4C below 26.63 pg/mg, whereas pregnant hind had HP4C both above and below this value, although almost 50% of them was below this threshold (Figure 18).

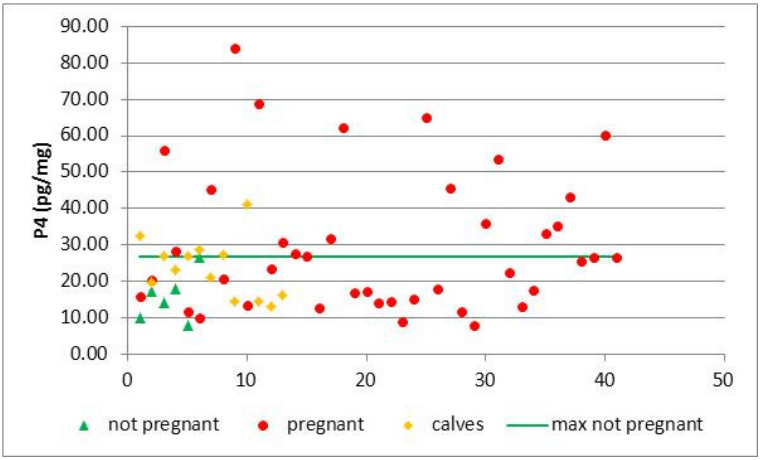

Figure 17: HP4C in not pregnant hinds, pregnant hinds, and calves. Green line indicates the maximum values (26.63 pg/mg) reached by not pregnant hinds, excluding calves.

HP4C significantly differs between pregnant and not pregnant individuals (Mann-Whitney U=44.0; $P<0.05$; Figure 18).

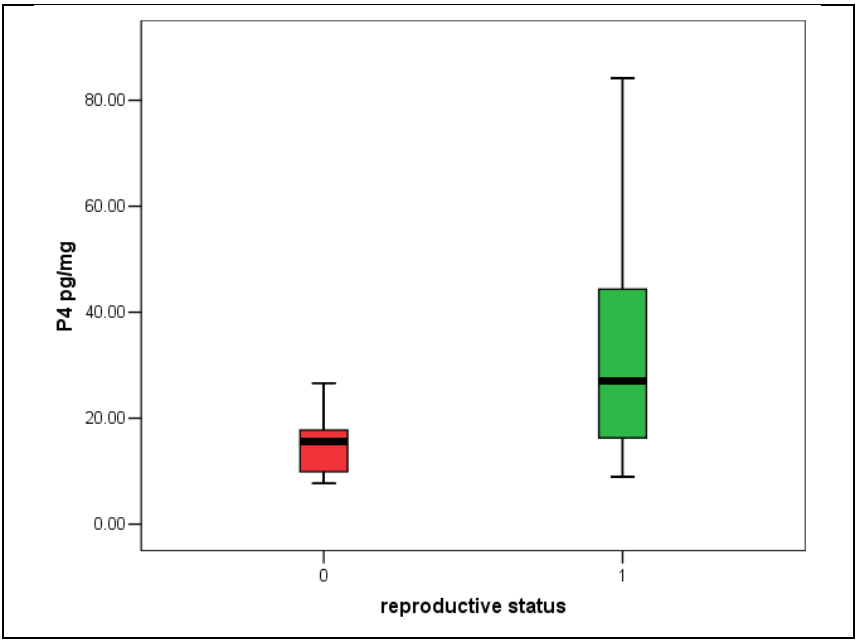

Figure 18: box plots of HP4C (pg/mg) in the 2 groups: 0=not pregnant females; 1=pregnant females.

No differences were detected between the individuals culled in January and in February (Mann-Whithney-U=153.0; *P*>0.05). In Figure 19, HP4C in pregnant and not-pregnant females older than 1,5 years for the two culling months are represented. Only three hinds (out of nine) culled in February fall below the threshold value (26.63 pg/mg).

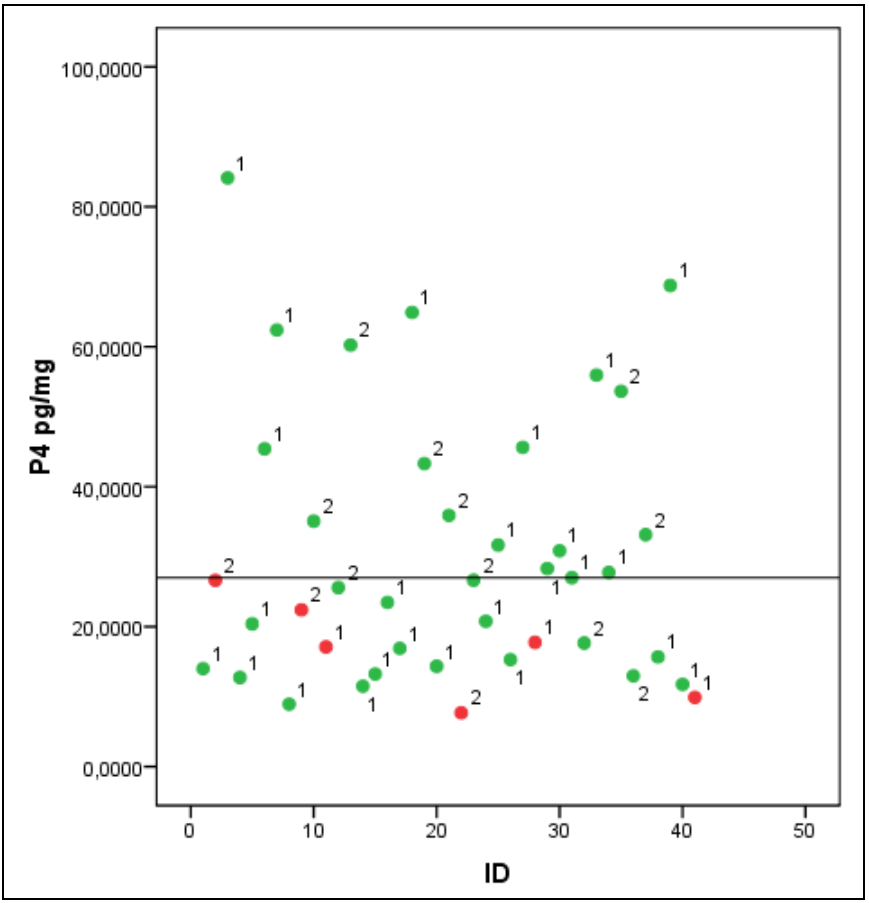

Figure 19: HP4C(pg/mg) in pregnant (green spots) and not pregnant (red spots) hinds (yearlings and adults). Numbers beside the spots indicate the month of culling (1=January; 2=February). The black line indicates the maximum HP4C reached by a not-pregnant individual.

A positive Spearman correlation was detected between HP4C and Hair Cortisol Concentration HCC (correlation coefficient=0.528; $P<0.001$).

The best logistic model, with the highest AIC value, is the one that includes only HP4C and KFI. This overall model is not significant; however, HP4C effect approaches statistical significance (P=0.06)..

3.5.4 Discussion

To our knowledge, this is the first study concerning the assessment of P4 concentrations in hair in free-ranging red deer, so our results represent the first reference values for this species.

A HP4C threshold value (26.63 pg/mg) was detected above which all the hinds are pregnant (Figure 17), even if this isn't sufficient to develop a feasible pregnancy test: some pregnant hinds, in fact, fall below this value. However, observing Figure 19, it's possible to note that just three pregnant hinds culled in February fall below the threshold value, and that two (out of three) not-pregnant hinds culled in February reached the highest values within the not-pregnant hinds. This may be due to the fact that P4 seems to accumulate in the hair during the whole pregnancy period (in pregnant hinds) or time by time with consecutive heats (in not-pregnant ones), and so the hinds culled in February have had a longer accumulation time with respect to the hinds culled in January (16-26 days more).

The finding of a threshold value of HP4C below which all the not-pregnant females are concentrated can be useful in discriminating, but it wasn't sufficient *per se*: for this reason we investigated the dependence of pregnant/not pregnant status from HP4C and KFI with the logistic analysis. The lack of significant results is probably due to several problems: i) a limited total sample, ii) biased for the small number of not-pregnant hinds, and, what is more, iii) referred just to a limited temporal period, reducing in that way the possibility of variability within the sample; but above all, iv) the fact that probably HP4C isn't by itself an indicator of reproductive status, needing to be

associated with other hormones quantification, as other Authors suggest (Willard *et al*., 1994). This may be probably because, in cervids, the highest P4 peak reached during the cycle is similar to plasmatic concentrations of P4 during most of the pregnancy (Thimonier and Sempere, 1989) (see paragraph 2.3.4); so some not-pregnant females in January-February after several heats, could have accumulated in hair a P4 concentration similar to the one of pregnant females.

The positive correlation between HP4C and HCC is not unexpected, and confirms previous findings about cortisol increase during pregnancy (Trainer, 2002).

In order to develop a more complex model to discriminate among pregnant and not-pregnant hinds and to provide a feasible pregnancy test, it may be interesting to evaluate HP4C variations together with other substances concentrations (e. g. oestradiol, oestrone sulphate, cortisol, pregnancy-associated glycoproteins, prostaglandins) in a greater number of samples, taking into account also sampling date and biometric variations. In fact biometric measures, especially body weight, can affect pregnancy status, as hinds are usually fertile only when they reach at least 70-75% of the adult expected weight (Kelly and Moore, 1977; Albon *et al.*, 1983; Albon *et al*., 1986; Langvatn *et al*., 1996; Mattiello and Mazzarone, 2010).

The possibility to easily and non-invasively assess the reproductive status of free-ranging large mammals should be an important tool in wildlife management for ecological consequences that reproductive performances play in population dynamics and for the current difficulties to investigate them on field.

3.6 Hair arsenic concentration assessment

3.6.1 Introduction

Arsenic toxicity is a global health problem affecting millions of people (Ratnaike, 2003). It is known to determine a number of alterations in the organism, among which renal lesions (see paragraph 2.4.2). Nephrotoxicity is due to the fact that urinary elimination is the main route of excretion and the proximal tubules are particularly sensitive due to their high reabsorptive activity (Madden and Fowler, 2000).

In 2011, a survey carried out in SNP showed how 48 animals in a sample of 68 individuals found dead presented renal lesions, in particular lythiasis (Figure 20) and tubulonephritis (Figure 21) (Bianchi *et al*., 2011).

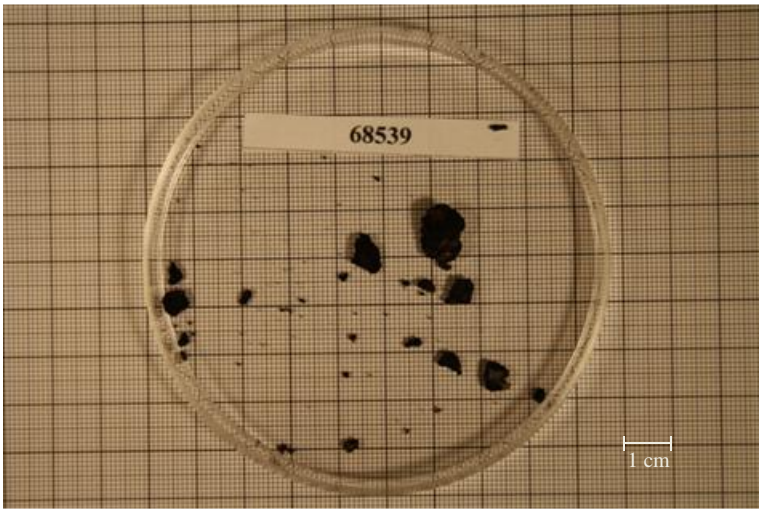

Figure 20: Lythiasis from red deer kidneys from SNP (photo: Istituto Zooprofilattico della Lombardia ed Emilia Romagna, Sez. Sondrio).

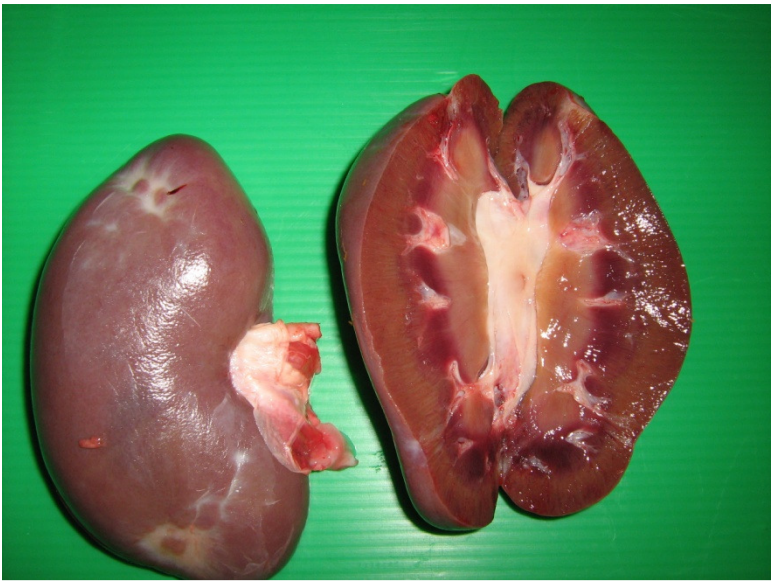

Figure 21: Tubulonephritis in red deer kineys from SNP (photo: Istituto Zooprofilattico della Lombardia ed Emilia Romagna, Sez. Sondrio).

The spatial behaviour of about 100 red deer in SNP have been monitored by radio-tracking for some years: the results allowed to define some sub-populations within the SNP territory (Pedrotti, *in verbis*). After the rut period, most of red deer stags reach their winter districts (when different from rut districts) and they establish themselves there until subsequent spring. In this phase they are highly sedentary and just some individuals can move (e.g. young males in dispersion) (Pedrotti, *in verbis*). From these remarks, it is possible to presume that during the control period in SNP period individuals were rather stable in one of the two orographic sides of Val Zebrù.

It is important to note that meat of culled red deer was destined for human consumption. In this way, virtually dangerous substances (e.g. heavy metals, or other pollutants, as arsenic) could have entered the

human food chain: in this context, the possibility to assess arsenic concentrations in culled animals could be an important tool in order to reduce health risks for consumers.

The present study aims are: i) to develop a method to assess arsenic concentration in red deer hair (hereafter: HAC=Hair Arsenic Concentration) samples; ii) to highlight possible correlations between HAC and kidney weight (used as indicator of possible renal problems) in red deer. The method for HAC assessment should be easy, cheap and quite rapid, in order to enable a quick assessment of the risk for human consumers of meat.

3.6.2 Materials and Methods

3.6.2.1 Samples collection

Hair Arsenic Concentration (HAC) was determined in 15 male red deer (4 calves, 4 yearlings, 6 adults) culled in January and February 2012 in SNP. On the basis of the results of previous studies on spatial behaviour in SNP (Pedrotti, *in verbis*), the samples were selected according to their provenience (Figure 22): 7 from the right orographic side of Val Zebrù (no arsenic in fonts = As-no) and 8 from the left orographic side (arsenic in fonts = As-yes). One individual culled in As-no side showed the highest arsenic concentration in our sample (12.8 µg/g) and it was considered as a male in dispersion, because of his sex and age (3.5 years). This individual was not considered for subsequent statistical analysis.

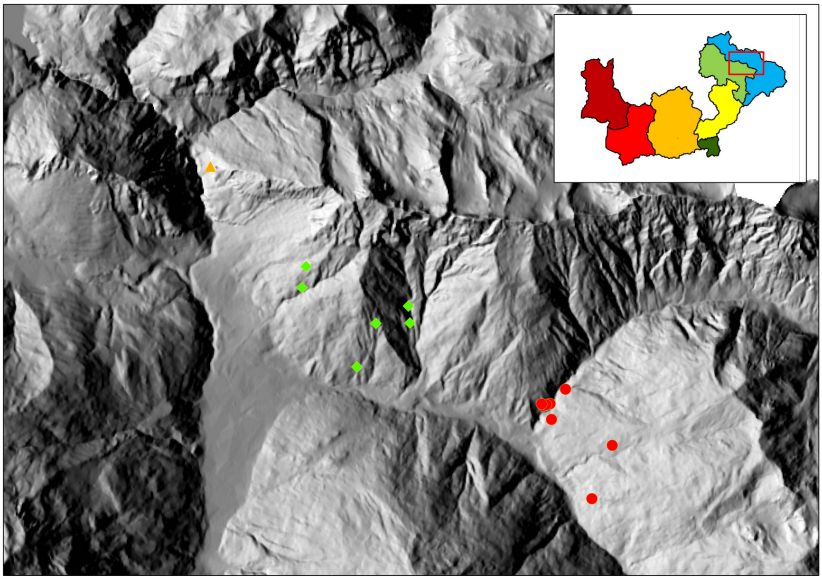

Figure 22: Localization of red deer culling on the two Val Zebrù sides. Red spots=As-yes; green squares=As-no; yellow triangle=outlier individual.

Few grams of hair were taken from the withers area of each individual. Hair was dried at room temperature, placed in paper envelopes and handed to the laboratory.

3.6.2.2 Hair arsenic assay

Approximately 0.5 g of the sample was digested with 3.5 ml of HNO3 and 1 ml H2O2 in microwave digestion system Milestone (USA). The temperature program was as follows: 2 min at 250 W, 2 min at 0 W, 5 min at 250 W, 8 min at 500 W and 5 min 750 W. The resulting solutions were cooled and diluted to 50 ml with deionized water. The entire procedure was checked for accuracy. Each analytical run also included standard reference material with known concentrations of As were carried out by using the same procedures. The clear solutions

were analyzed by ICP-OES SPECTRO CIROS M CCD-ICP with pneumatic nebulizer.

For calibration, standard solution were prepared from the stock standard solution of 1000 ng/ml by dilution. The ranges of the calibration curves (6 points) were selected to match the expected concentrations (0–15 µg/g) for the element of the sample investigated by ICP-OES. Linearity was checked in the range of 0–1000 µg/g. Detection limits were calculated as the concentrations of an element that gave a signal equal to three times the standard deviation of a series of five successive measurements of the blank solution at the element peak.

3.6.2.3 Statistical analysis

The concentration data, expressed in µg/g, were stored using MS Excel 2010. Statistical analysis were performed with SPSS 13.0 software. Graphs were performed with SPSS 13.0 software or MS Excel 2010.

Differences in HAC and in kidney weight (KW) between the two groups (As-no and As-yes) were tested with a Mann-Withney U-test. Differences in HAC among the three age classes (Calves=0.5 years, Yearlings=1.5 years, Adults≥2.5 years) were tested with a Kruskal-Wallis test. Partial correlations, corrected respectively for weight and for age class, were calculated between KW and HAC.

A residual probability value of 5% (P=0.05) was adopted as the minimum level of significance.

3.6.3 Results

The minimum HAC observed was 2.26 µg/g; the maximum was 10.55 µg/g. Median HAC resulted higher in As-yes group than in the As-no one (Mann-Whitney-U=3.0; $P<0.01$) (Figure 23).

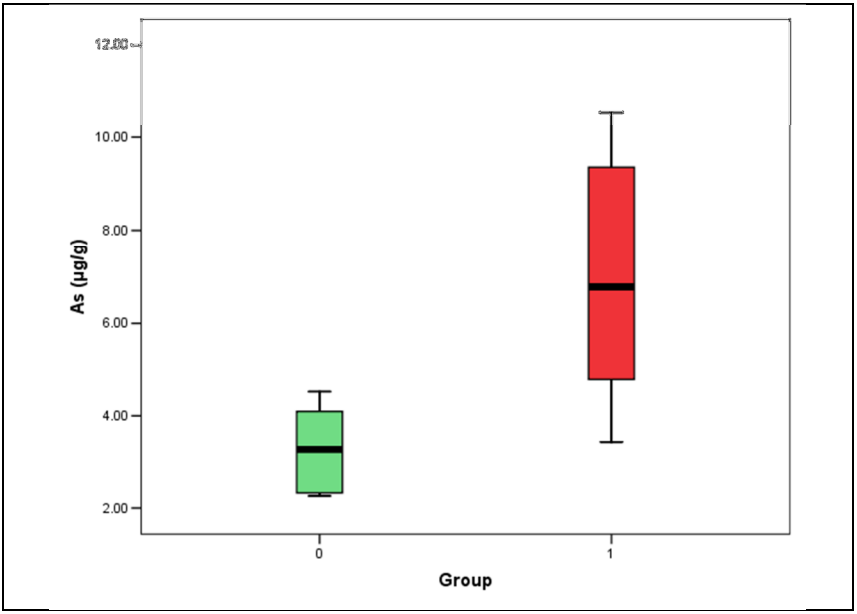

Figure 23: box plots of HAC in the 2 groups: 0=no arsenic in fonts (As-no); 1=arsenic in fonts (As-yes).

No differences in HAC were observed among the three age classes (Chi-square=3.27; $P>0.05$).

No differences were detected in KW between the two groups (Mann-Whitney-U=10.0; $P>0.05$). Mean KW in each age class, for the two sides of Val Zebrù, are showed in Table 16.

		Calves	Yearlings	Adults	Total
As-yes	**n**	1	2	5	8
	mean KW (g)	119	188.7	225.56	203.025
As-no	**n**	3	2	1	6
	mean KW (g)	145.4	171.15	179.1	159.6
Total	**n**	4	4	6	14
	mean KW (g)	138.8	179.93	217.82	184.41

Table 16: mean KW (g) for each age class in the two sides of Val Zebrù. As-yes=arsenic in fonts; As-no=no arsenic in fonts.

Significant partial correlations, respectively corrected for carcass weight (correlation=0.633; $P<0.05$), and for age classes (correlation=0.568; $P<0.05$; Figure 24) were detected between KW and HAC.

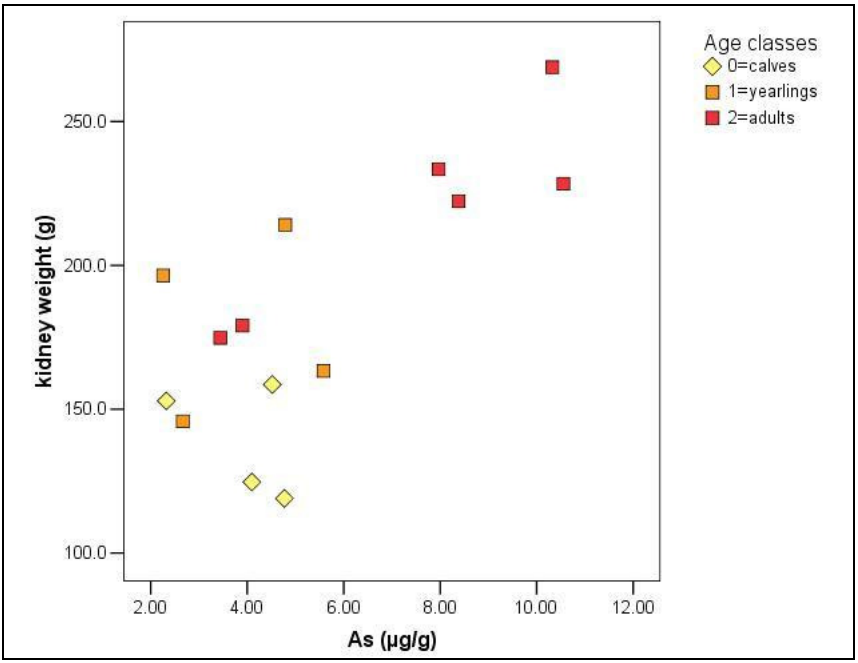

Figure 24: correlation between kidney weight (g) and HAC (µg/g), corrected for age classes (partial correlation=0.568; $P<0.05$).

3.6.4 Discussion

Because the last moult occurred in September-October, and the culling was in January-February, HAC reflects the arsenic accumulation over a maximum of 3-4 months. Notwithstanding this relative brief period, a significant difference in HAC was detected between the two groups (As-yes and As-no). This is probably due to an important water contamination, that allows rapid accumulation in animals drinking tainted water and eating on contaminated pastures.

Renal lesions detected in previous studies in red deer found dead in SNP (Bianchi *et al*., 2011) could have been related to arsenic contamination, but no information was available about arsenic contamination in those individuals. Unfortunately, the results of histologic analysis of the kidneys of red deer used for our research are not available yet. However, KW could be expression of renal disturbs and the correlation between HAC and KW, corrected for age and sex class, suggests that a relation could exist between arsenic contamination and renal pathology. More detailed investigations are certainly necessary, as histologic examinations of renal lesions (that are currently still being carried out), and determination of arsenic concentration in kidney or other tissues.

The importance to detect arsenic contamination in wild red deer and in other culled wild ungulates is also due to the possibility of meat contamination. Recently, wild animals meat consumption has been increasing in Italy and in other European Countries because of its large availability, ensuing the general increase of wild ungulates populations and culling (Monaco *et al*., 2003; Milner *et al*., 2006; Carnevali *et al*., 2009; Ramanzin *et al*., 2010). In order to ensure meat safety, the assessment of heavy metals and other environmental

contaminants, as arsenic, cannot be neglected, because of the possible risks for public health.

The consumption of meat from wild culled animals can actually be a marginal, even if increasing, problem; nevertheless, also domestic food producing animals (milk and meat) grazing on contaminated areas could, in turn, be contaminated, becoming a further risk for public health.

HAC assessment could be an easy, rapid, and low cost screening method and it could be useful i) in wildlife management programs, in order to investigate wild population health status; ii) in environmental studies, to assess arsenic presence in wild species and to determine risks for humans; and not last, in public health programs iii) to estimate contamination risks linked to wild animals meat consumption; iv) to assess arsenic contamination in food producing animals living in contaminated areas.

4 Conclusions

The analysis of hormones or other substances in hair constitutes a highly promising and reliable method for assessment of substances secretion over extended periods of time in free-ranging red deer.

In particular, our findings suggest that i) HCC provides a good index of long-term HPA axis activity and allostatic load in individuals from areas with higher red-deer density, higher anthropic disturbance, and harder environmental conditions; ii) using HP4C, together with other sexual hormones concentrations in hair and biometric measures, may be possible in the future to develop a reliable and easy pregnancy test for free-ranging red deer; iii) HAC (and other micro-elements or heavy metals concentrations in hair) could be analysed, not only in order to assess wild populations status, but also to control wild animals contamination, in biomonitoring investigations or in health programs.

The difficult to collect samples from free-ranging wild large mammals in general, and from red deer in particular, is known. Problems are related to: i) field conditions that often are hard and adverse (bad weather conditions, difficult in reaching the capture site); ii) capture and handle the animals (risks for the animals, but also for the operators safety); iii) collection and conservation of the sample (necessity of experienced operators and specific devices); iv) elevated costs for capturing wild animals; and v) limited number of captured individuals. Hair samples can be easily collected from culled deer; moreover, the possibility to collect hair samples from free-ranging living red deer using hair snares (Belant *et al.*, 2007), extends even more the applications spectrum of the methods we investigated: the lack of the necessity to capture and handle animals would enable the researchers to collect a great number of samples from a population

and that, together with the assessment of substance in hair, would allow wide spectrum investigations on free-ranging red deer populations status.

Moreover, our investigations could provide a basis for developing similar assays in other free-ranging species, remembering that for each species validation of the method and of the results will be request.

In conclusion, the assessment of hormones and micro-elements in the hair seems to be an interesting tool for future wild species management, although further investigations are certainly request to detect definite reference values. Such a non-invasive method to assess the populations status could have an important role in free-ranging large mammals research and management, reducing sampling efforts for researches and disturbance and risks for animals, making wide spectrum investigations on free-ranging population status achievable.

5 References

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6 Appendices

6.1 List of Abbreviations

Abbreviation	Significance
SAS	Sympathetico-Adrenomedullary System
HPA axis	Hypotalamic-Pituitary-Adrenocortical axis
LUC-NE	Nerve Fibres of Locus Coeruleus Region
CRH	Corticotropin-Releasing Hormone
ACTH	Adrenocorticotropic Hormone
GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
IGF-I	Insulin-like Growth Factor
CNS	Central Nervous System
NEFA	Non Esterificated Fatty Acids
GCS	Glucocorticoids
CA	Comprensorio Alpino (Alpine Hunting District)
CA-MO	CA Morbegno
CA-SO	Ca Sondrio
CA-AV	CA Alta Valle
SNP	Stelvio National Park
HCC	Hair Cortisol Concentration
HP4C	Hair Progesterone (P4) Concentration
HAC	Hair Arsenic Concentration
BL	Body Length
FL	Foot Length
WH	Withers Height
JL	Jaw Length
CW	Carcass Weight
KW	Kidney Weight
KFI	Kidney Fat Index

6.2 List of Tables

6.3 List of Figures

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