PhD Thesis:

Study of territorial behaviour and stress for the improvement of European wild rabbit restocking programs.

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TITULO: *STUDY OF TERRITORIAL BEHAVIOUR AND STRESS FOR THE IMPROVEMENT OF EUROPEAN WILD RABBIT RESTOCKING PROGRAMS.*

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[…] Pensé enseguida en el ansiado conejo,
dos o trescientos metros más abajo,
olisqueando la rala hierba, muy activo,
enamorado tal vez, con una familia que fundar,
proyectos de carrera,
ojos de ágata y pelaje de seda,
orejas transparentes y vivo hocico,
una obra maestra de la naturaleza, también…
(Daniel Pennac 1995)

[…] las ratas son el paradigma. No olvides eso y nunca te equivocarás demasiado. Las ratas son el paradigma.
(Tom Sharpe, 1976)

Gracias…

A Francis el primero, por darme esta segunda oportunidad.

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ABSTRACT

The European rabbit (*Oryctolagus cuniculus* L.) is a keystone species that is native to the Iberian Peninsula and whose populations have undergone a dramatic decline in abundance in their natural ranges throughout the second half of the 20th century. The economic and ecological importance of the wild rabbit has now led to the need to perform management actions aimed at boosting the remaining populations and establishing new ones, particularly in Mediterranean areas. Restocking actions are one of the handiest management tools, and scientific research whose objective is to define and solve the major methodological problems is abundant in literature. However, the majority of research has focused on factors which are extrinsic to wild rabbits, such as predation, diseases and habitat, while neglecting other factors that are intrinsic to wild rabbit biology. The principal aim of this PhD thesis is to improve the efficiency of wild rabbit restocking programs by taking into account intrinsic aspects of rabbit biology such as social behaviour and stress. In order to do so, I have performed five experiments whose intention has been to test Fecal Near Infrared Reflectance Spectroscopy and indigestible faecal markers as cheap, easy, user-friendly and non-invasive tools by which territorial marking by wild rabbits can be studied; I have also measured restocked wild rabbit populations' responsiveness to acute stressors in a non-invasive manner as a predictor of population growth during the breeding season; I have additionally studied the density-dependence phenomenon inside wild rabbit restocking plots, with the aim of providing a better management of these plots and improving their efficiency as extensive breeding sites; and finally, I have performed a supplementary experiment with wild-type rats with the intention of attaining a better understanding of the relationship between physiological stress and territorial behaviour. The first four chapters are framed

within the project to improve the black vulture's (*Aegypius monachus*) habitat in Córdoba province, which was managed by the Andalusia Government's Environmental Agency. The last chapter was developed in the University of Groningen (The Netherlands), in collaboration with the Department of Behavioural Physiology. My results suggest that both Fecal Near Infrared Reflectance Spectroscopy and indigestible faecal markers are suitable tools with which to study wild rabbit territorial marking behaviour. In addition, the responsiveness to acute stressors is suggested to be a feasible and non-invasive predictor of wild rabbit population growth. Density-dependence is described in wild rabbit restocking plots, and optimum rabbit abundances per hectare are proposed in order to increment the productivity within the enclosures. Lastly, the experiment performed at the University of Groningen using wild-type rats suggests the importance of the physiological stress response in the development of social relationships based on territoriality and dominance, and hence points to the need for the further study of stress mechanisms in the context of rabbit sociobiology.

INTRODUCTION

My case study: the European rabbit.

The European rabbit (*Oryctolagus cuniculus* L.) is a native key species in the Iberian Peninsula, and is one of the most widely distributed living lagomorphs throughout the world. The Iberian Peninsula is the most probable ancestral area of the species: the first fossil records have been located in southern Spain during the Mid Pleistocene period (0.6 Ma), and, for most of its history, the European rabbit has been confined to this plesiocoric area, where it has constituted an important trophic source for the survival of large predators. It has diverged into two subspecies: *O. c. cuniculus* and *O. c. algirus* (Branco et al., 2000). The current worldwide distribution of the species is a result of one of the most remarkable human-mediated geographical expansions among mammals: the European rabbit has successfully colonized North Africa, Europe, South America, Australia, New Zealand and hundreds of islands all over the world as a result of human transport, induced changes in habitat and its domestication as a source of meat, fur and wool (Ferrand, 2008, López-Martínez, 2008a).

The 20th century population crash.

Regretfully, the wild rabbit populations in the Iberian Peninsula have undergone a massive decline during the 20th century. This decline was already underway during the first half of the century as a result of habitat loss and fragmentation, and human-induced mortality (Ward, 2005), and this could have, in part, induced the high impact of disease (Calvete, 2006) when myxomatosis began to attack the Iberian rabbit populations in the 1950s (Muñoz, 1960) and killed over 90% of the rabbits. Rabbit populations began to show signs of recovery during the 80s, but the

appearance of a new virus - Rabbit Haemorragic Disease (Argüello et al., 1988) - devastated the remaining populations, reducing them to as few as 5% of the number of rabbits that had existed prior the incidence of these viral diseases (Ward, 2005). The later increase in opportunistic predators may have prevented the populations from recovering their initial levels - the "predator pit" hypothesis (Trout and Tittensor, 1989, Pech et al., 1992).

The population recovery and changes in the distribution range.

However, rabbits have managed to recover their populations in the Iberian Peninsula, although unevenly, since a shift in their distribution range has been observed over the last few decades (Figure 1), with high density populations restricted to disperse patches of high quality habitats. The new optimum sites are in agricultural areas, in dry wood crops and grasslands (Vargas et al., 2007, Farfán et al., 2008). The changes in land use that have occurred in the Iberian Peninsula over the last few decades have led to geographical differences in rabbit favourability (Delibes-Mateos et al., 2010), with the subsequent paradoxical effects (Lees and Bell, 2008, Delibes-Mateos et al., 2011).

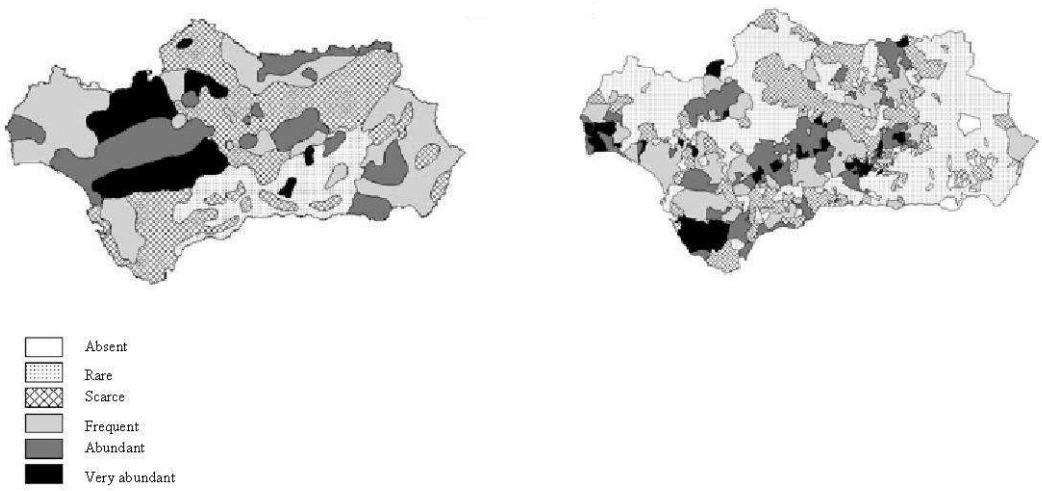

Figure 1. Changes in abundance and distribution of the European rabbit (*Oryctolagus cuniculus*) in Andalusia, southern Spain, from the 1960s (left) to the present (right). Modified from (Delibes-Mateos et al., 2009).

The rabbit paradox.

The European rabbit is simultaneously classified as a Near-Threatened species in the Red List of Vertebrates of Spain according to IUCN criteria (Villafuerte and Delibes-Mateos, 2007), and regarded as an agricultural pest (Barrio, 2010). As a keystone species in the Iberian Peninsula (Delibes-Mateos et al., 2007), it influences plant communities (Eldridge and Simpson, 2002, Eldridge et al., 2006), it is an important part of the diet of at least 39 predators, including highly endangered species such as the Iberian Lynx (*Lynx pardinus*) and the Imperial Eagle (*Aquila adalberti*) (Delibes and Hiraldo, 1981, Delibes-Mateos et al., 2007), and it is also an important ecosystem engineer (Gálvez, 2008). The European rabbit is also a favourite prey for human beings, since it is the most important small game species in Spain (Delibes-Mateos et al., 2008b). The decrease in rabbit numbers has therefore led to important cascading effects in the functioning of the Mediterranean ecosystem, with serious ecological and economical consequences (Delibes-Mateos et al., 2008a). However, and paradoxically, the recovery of rabbit populations in agricultural croplands and the

damage to crops caused by them (Barrio et al., 2010b) has led to their persecution as an invasive pest species within their native range (Delibes-Mateos et al., 2011).

Conservation value and population management.

The recovery of rabbit populations in agricultural areas has led to the assumption that rabbit populations may get to be recovered in other spots, and the management efforts of both conservationists and hunters have therefore converged upon increasing rabbit numbers in a wider range. The most frequently used management strategies have been habitat management, reduction of hunting pressure, predator control, restocking and vaccine programs (Angulo, 2003). Rabbit translocations have particularly increased in the Iberian Peninsula over the last few decades (Delibes-Mateos et al., 2008a). The most frequent procedure is the translocation of captured wild rabbits to predator-exclusion fenced plots (Ferreira and Delibes-Mateos, 2010), which improves the restocking efficiency by reducing the short-term mortality rates (Calvete et al., 1997, Calvete and Estrada, 2004) and increasing the long-term population growth (Rouco et al., 2008). Various projects, such as the Iberian Lynx LIFE project, have been responsible for building more than 260 rabbit fenced plots since 2002 (Gil-Sanchez, 2011). These fenced plots may have two aims: the establishment of supplementary feeding stations for predators (López-Bao et al., 2008) or the creation of centres for the dispersion of rabbits. In the latter case, the enclosures are breeding systems which are protected against terrestrial predators, and their aim is to increase rabbit abundance, thus enabling the rabbits to then spread and colonize the surrounding areas (Rouco et al., 2008).

The role of social behaviour in restocking programs.

To date, the efforts made to improve translocations have been directed towards extrinsic factors such as predation (Rouco et al., 2008, Rouco et al., 2010), viral diseases (Calvete et al., 2004b, Calvete et al., 2005), or the availability of shelter and food (Cabezas and Moreno, 2007, Fernández-Olalla et al., 2010, Cabezas et al., 2011). However, research efforts that consider intrinsic factors such as social interactions are still scarce (but see (Von Holst et al., 1999, Letty et al., 2000, Rödel et al., 2006, Cabezas et al., 2007, Cabezas et al., 2011). Gregariousness, social grouping, territoriality and social dominance are integrant parts of the biology of the wild rabbit, and as such they influence breeding and the survival of the individuals within the group (Mykytowycz and Goodrich, 1974). While group-living in wild rabbits appears to have arisen as a response to resource localization, its maintenance could involve subsequently evolved social features (Cowan, 1987), since wild rabbit social groups consist of hierarchies (Mykytowycz, 1958, Mykytowycz and Gambale, 1965). There are separate linear ranking orders for bucks and does, and the top-ranking buck dominates the whole colony. Moreover, during the breeding season, territorial behaviour is enhanced and top-ranking bucks and does defend their warrens and exclude the subordinate individuals (Mykytowycz, 1958, Mykytowycz and Gambale, 1965), and this may therefore limit their reproductive performance.

Hence, in order to attain a better understanding of the social mechanisms that have limited the success of rabbit restocking programs, I have focused my research on territorial scent marking, the relationship between territorial behaviour and stress physiology, and the possible effects of these intrinsic factors upon the growth of restocked wild rabbit populations. All of these issues are described in greater depth in the following paragraphs.

Scent marking.

Social interactions of wild rabbits are in part maintained by olfactory communication. In the wild rabbit, odour participates in the maintenance of gregariousness (Mykytowycz and Goodrich, 1974), has a role in territorial behaviour (Mykytowycz, 1962, Mykytowycz, 1964), enhances territorial confidence by permeating the territory (Mykytowycz et al., 1976), and separates and identifies social groups (Mykytowycz, 1962). In social signalling by skin glands, the strength of the dominant animals depends on their ability to communicate socially (Mykytowycz and Goodrich, 1974). The individual's rank is consequently characterized by the size and intensity of secretion of different pheromone-secreting glands (Mykytowycz, 1964). Rabbits have three major skin glands whose function is that of social communication: the inguinal gland, the chin gland and the anal gland. The secretion of the inguinal glands would appear to be associated with individual identification and sexual attraction (Mykytowycz, 1966); the chin-marking of rabbits that are external to the group seems to facilitate their acceptance by that group (Mykytowycz, 1962), while the rabbit's territorial marking is particularly associated with its anal glands (Mykytowycz, 1964): the faecal pellets are coated with their secretions (Mykytowycz and Gambale, 1969) and are deposited in latrines (Sneddon, 1991) which serve to delimit territories (Mykytowycz et al., 1976).

Territoriality.

Latrines identify the individuals with their territories and warn strangers entering them, they contribute to the maintenance of the integrity of the colony, and are principally established by the dominant buck (Mykytowycz and Gambale, 1969) (Figure 2). A better knowledge of territoriality may be of great use in improving the success of

restocking (Moreno et al., 2004, Letty et al., 2008). However, territories vary according to the availability of food, space, population density or habitat, since the intensity of territorial behaviour is conditioned by the trade-off between the cost of defending a territory and the benefits obtained from resource monopolisation (Maher and Lott, 2000). This variability makes the monitoring of rabbits' territories and territorial behaviour difficult. To date, territoriality in rabbits has been studied via latrine distribution by direct visual observation (Mykytowycz and Gambale, 1969) or latrine mapping (Monclús and De Miguel, 2003). However, the results of these studies are limited by the laboriousness of the mapping and the difficulty of observing rabbits in the wild. What is more, while the operational definition of latrines (Virgós et al., 2003) permits comparison among different studies, the social communication function of latrines (Mykytowycz, 1964) should also be tested for a better understanding of the social behaviour of the species.

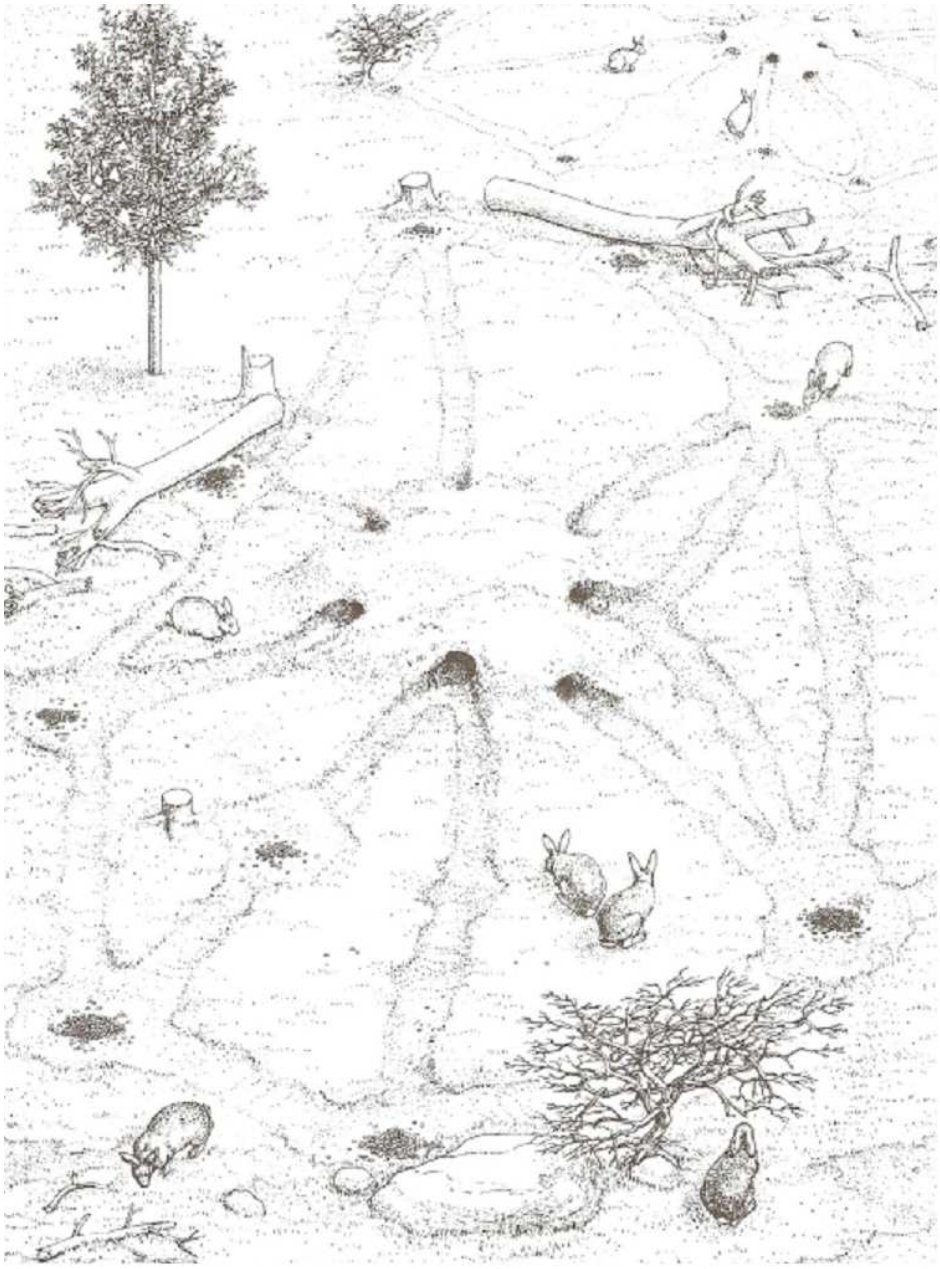

Figure 2. Latrine distribution in a European rabbit colony. From (Mykytowycz, 1964).

Social aggression and stress.

Aggressive behaviour has evolved as one of the most efficient means of competition in social species, which ensures individual survival and genetic success when resources are limited. As such, virtually all species have a neural system and a physical constitution that makes them suitable for performing it (Haller and Kruk, 2006). When aggression is directed towards the monopolisation of limited resources

through the defence of a territory, it becomes a major component of territorial behaviour (Maher and Lott, 1995, Maher and Lott, 2000). Much of the aggression neurocircuitry overlaps with the hypothalamo-pituitary-adrenal (HPA) axis (Summers and Winberg, 2006), for aggression is both a stressor (Kruk et al., 2004) and one of the most immediate mechanisms by which stress enhances survival (Haller et al., 1998a, Mikics et al., 2007). The stress physiology is therefore an important component of social interactions, and should not be neglected when social species are studied.

The physiological stress response.

The HPA axis is a major evolutionary mechanism with which to maximize fitness through stress management (Selye, 1950, Blas et al., 2007). The perception of a stressor (Romero, 2004) stimulates the secretion of catecholamines from the sympathetic nervous system, and starts a hormone cascade in the HPA axis which initiates and orchestrates the emergency life history stage within seconds to hours (Wingfield et al., 1998): the hypothalamus releases the corticotropin releasing factor (CRF), which induces the pituitary gland to secrete the adrenocorticotropic hormone (ACTH), which will, in turn, activate the adrenal glands and induce them to release glucocorticoids (GC) into the blood torrent (Figure 3). This hormone cascade is associated with simultaneous endocrine changes, which are expressed in the target issues within seconds to minutes (increased cardiovascular tone, immune activation, energy mobilization, increase of cerebral blood flow and glucose utilization, enhanced memory consolidation, loss of appetite, inhibition of reproductive physiology and behaviour, vasoconstriction). It precedes the slower GC effects, which, upon joining different kinds of corticosteroid receptors, will mediate changes in the structure and neurohistochemistry of the brain which will then condition subsequent stress responses

in addition to enhance the ongoing stress response, prevent it from overshooting and contribute to the recovery from it (Sapolsky et al., 2000, de Kloet et al., 2005). However, when the stress response is not adequately terminated and sustained elevations of GCs occur, the stress becomes chronic. The physiological cost of maintaining the homeostasis in these conditions is high: immunosuppression (Moynihan and Ader, 1996), impaired cognitive functions (de Kloet et al., 2005), illnesses related to the digestive system (Taché et al., 2001), loss of body mass (Cabezas et al., 2007) or impaired reproductive performance (Von Holst, 1998). Stress may therefore play an important role in animal management plans. Moreover, in addition to the negative effects of a sustained elevation of GCs, there is a close, though variable, relationship between non-pathological GC levels and fitness (Bonier et al., 2009), and although the acute stress reaction is assumed to be adaptive in nature, optimal acute stress reactions remain unknown (Breuner et al., 2008).

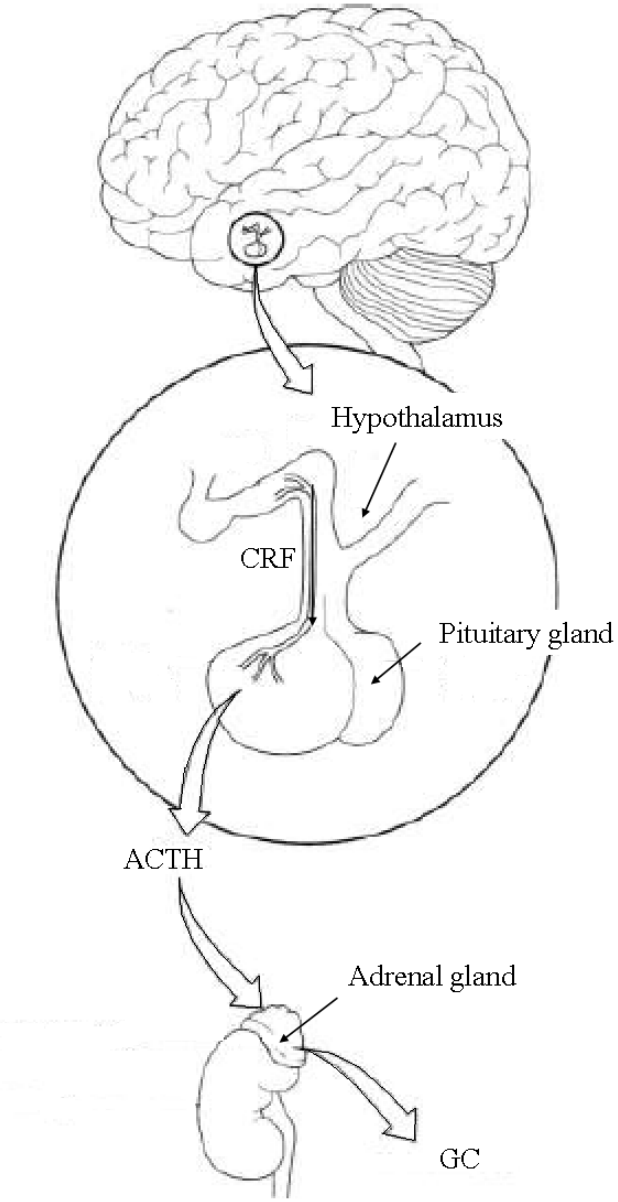

Figure 3. Hypothalamus-Pituitary-Adrenal axis, modified from (Sapolsky, 1994).

Stress and translocations.

Conservation biologists are using progressively more physiological techniques in order to elucidate the causal mechanisms underlying conservation problems and to monitor the efficacy of management strategies (Wikelski and Cooke, 2006). One of the endocrinological approaches to the conservation of wild animals is the monitoring of stress levels through GC analyses (Busch and Hayward, 2009). Such an approach is advisable for restocking actions, since the translocation process is a source of stress

(Dickens et al., 2010), and the success of the translocation is affected by the translocated individuals' stress levels (Letty et al., 2000, Cabezas et al., 2007, Teixeira et al., 2007, Letty et al., 2008, Cabezas et al., 2011). However, the monitoring of stress levels in translocated animals is far from being a routine analysis.

The productivity of restocking plots.

One of the foreseeable consequences of neglecting the social organization and stress physiology of the wild rabbits is a reduction in the efficiency of extensive rabbit breeding systems, with the associated waste of effort and economic resources, and the failure in the sustainable production of rabbits. Social interactions within the rabbit restocking enclosures (Letty et al., 2008) limit the population growth, since individuals compete for social rank and territory as substitutes for key resources, and these behavioural traits are density-dependent (Wynne-Edwards, 1959). Indeed, in rabbit populations, fecundity and population growth decrease as density increases (Myers and Poole, 1962, Myers and Poole, 1963), and lower reproduction rates and a higher mortality have been observed in high rabbit densities (Rödel et al., 2004a, Rödel et al., 2004b). Social interactions have also been regarded as an important source of stress in low-ranking wild rabbits (Von Holst et al., 1999), and higher levels of chronic stress have been described in higher densities of other mammal species (Rogovin et al., 2003, Li et al., 2007).

Non-invasive analyses.

When individual traits condition population dynamics, the usual approach is to monitor the individuals (Romero and Wikelski, 2010, Carter et al., 2012, Galaverni et al., 2012). However, for social species, data from individuals are not sufficient to

determine the group behaviour, and it is therefore necessary to seek other techniques with which to study group characteristics. What is more, when studying species of conservation value, a non-invasive approach is advisable. The analysis of faeces can provide useful information about both individuals and the environmental factors surrounding them (Tolleson et al., 2005), whilst the pooling effect in the digestive tract signifies that the information is integrated over a period of time (Touma et al., 2004). Faeces have been used to indicate abundance (Taylor and Williams, 1956, Putman, 1984), in the analysis of diet (Martins et al., 2002), in the evaluation of parasite burden (Gobert et al., 2005), and to indicate stress levels (Cabezas et al., 2007), reproductive state (Rolland et al., 2005) or territorial marking (Mykytowycz, 1964). However, the analysis of chemical components implies highly complex extractions and purifications, together with electrophoresis, chromatographs, mass spectrometry or immunoassays (Hesterman and Mykytowycz, 1968, Goodrich and Mykytowycz, 1972, Goodrich et al., 1981, Heistermann et al., 1993, Sun and Müller-Schwarze, 1998, Zhang et al., 2002, Cabezas et al., 2007), and these analyses are costly in terms of time, effort and money, in addition to requiring a specialised preparation.

THESIS STRUCTURE AND PRINCIPAL AIMS

The principal aim of this thesis is to improve the efficiency of wild rabbit restocking programs by taking into account intrinsic aspects of rabbit biology such as social behaviour and stress. With this objective in mind, the thesis has been structured in five independent chapters in scientific paper format, each of which addresses one specific question concerning methodological, management and experimental issues. The goals of the different chapters are:

1. To introduce and test the feasibility of the Faecal Near Infrared Reflectance Spectroscopy as a new, easy, cheap and non-invasive method with which to study the territorial chemical signalling in wild rabbits.
2. To test the feasibility of non-digestible faecal markers in the identification of the territories of wild rabbit social groups.
3. To measure the responsiveness of restocked wild rabbit populations to acute stressors in a non-invasive manner, and to study the relationship between the responsiveness and the productivity of those populations.
4. To analyse the effect of rabbit abundance upon the productivity of rabbit populations within restocking enclosures.
5. To test the effect of the blockade of the physiological stress response upon the escalation of territorial aggression.

STUDY AREA AND GENERAL METHODS

This study was framed within the project to improve the black vulture's (*Aegypius monachus*) habitat in Córdoba province, which was managed by the Andalusia Government's Environmental Agency. All the enclosures were built according to the same design and maintained by the same staff throughout the duration of this research. The process of capture, transport and subsequent release inside the enclosures was also carried out by the Environmental Agency. The rabbit populations inside the enclosures were monitored through the use of monthly rabbit pellet counts at fixed points (Fernández-de-Simón et al., 2011) between September 2008 and July 2012.

The studies described in Chapters 1 to 4 were developed in 26 wild rabbit restocking enclosures of 1.95 ± 0.35 (SE) ha, distributed among 7 big-game hunting estates located in central Sierra Morena, Córdoba, Southern Spain (38º 5' N, 5º 16' W) (Figure 4). This area is very important both for conservation and sport-hunting, and currently contains low-density rabbit populations, although rabbits were abundant in the past. The main ecosystems in this study area include Mediterranean scrubland, pine forest and oak savanna (dehesa), and the climate is dry Mediterranean. Soils are granitic and hard to dig, so warren availability was expected to be limited in the area. The principal rabbit predator species coexist in the study area, with the exception of the Iberian Lynx (*Lynx pardinus*): terrestrial predators such as the red fox (*Vulpes vulpes*), the Egyptian mongoose (*Herpestes ichneumon*), the marten (*Martes foina*), the genet (*Genetta genetta*) and the wildcat (*Felis silvestris*), and raptor species such as the Spanish imperial eagle (*Aquila adalberti*), the golden eagle (*Aquila chrysaetos*), the eagle owl (*Bubo bubo*), the bonellis eagle (*Hieraetus fasciatus*), the booted eagle (*Hieraetus pennatus*) and the buzzard (*Buteo buteo*).

Figure 4. Location of the rabbit restocking enclosures within the hunting estates.

All the enclosures were built to exclude ungulates and terrestrial predators by using 2 m fences with 0.5 m buried underground and two electric wires at different heights. Refuges (heaps of branches) and artificial warrens (2-6 per ha, constructed with pallets and rocks, and covered with earth and branches) were provided for shelter and nesting, and water and food (a grain and rabbit pellet mixture) were supplied *ad libitum* during the entire study period.

The restocked rabbits were captured in an agricultural area located in the south of Córdoba province (37º 34’ N, 4º 37’ W), which contains medium to high rabbit densities. This area is characterized by a dry Mediterranean climate and calcareous soils dedicated to olive groves, vineyards and cereal fields. The rabbits were randomly

released inside the enclosures on the same day as their capture, within the natural distribution area of the subspecies *Oryctolagus cuniculus algirus* (Branco et al., 2000) and in the same sex-ratio as in capture, and they were not subsequently submitted to any kind of artificial selection. In 20 of the enclosures, the rabbits were released during autumn 2008, while in the other 6, the rabbits were released during autumn 2010.

The experiment on territorial aggression described in Chapter 5 was performed in the Department of Behavioural Physiology at the University of Groningen, The Netherlands. The case study in this work was the Wild Type Groningen rat (*Rattus norvegicus*), the ancestors of which were trapped in the wild and subsequently bred in a laboratory for 49 generations.

CHAPTER 1

Near Infrared Reflectance Spectroscopy – NIRS – for the Detection of Territorial Marking in European Rabbit Pellets.

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Introduction

The analysis of faeces can provide useful information about both individuals and the environmental factors surrounding them (Tolleson et al., 2005). Faeces have been used to indicate abundance (Taylor and Williams, 1956, Putman, 1984), in the analysis of diet (Martins et al., 2002), in the evaluation of parasite burden (Gobert et al., 2005), and to indicate stress levels (Cabezas et al., 2007), reproductive state (Rolland et al., 2005) or territorial marking (Mykytowycz, 1964). However, the analysis of chemical components implies highly complex extractions and purifications, together with electrophoresis, chromatographs, mass spectrometry or immunoassays (Hesterman and Mykytowycz, 1968, Goodrich and Mykytowycz, 1972, Goodrich et al., 1981, Heistermann et al., 1993, Sun and Müller-Schwarze, 1998, Zhang et al., 2002, Cabezas et al., 2007) and these analyses are costly in terms of time, effort and money, in addition to requiring a specialised preparation.

In contrast to traditional analyses, F.NIRS allows the analysis of faeces to be carried out more rapidly, easily and cheaply through the absorption of radiation in a range of between 700 and 2500 nm through chemical links, principally those which include C, H, N and O. Faecal NIRS -F.NIRS- has been used to study the diet, physiological state, sex, reproductive state, parasite burden and ecology of free-range

herbivores (Boval et al., 2004, Tolleson et al., 2005, Tolleson et al., 2007, Dixon and Coates, 2009). However, the chemical components related to territorial marking have not as yet been studied with F.NIRS.

Social interactions, such as territoriality, are elements which are relevant to the biology of gregarious species, and their influence on reproduction and survival affects population density (Wynne-Edwards, 1959). This study uses the wild rabbit (*Oryctolagus cuniculus* L.) as an experimental model, and attempts to determine the validity of the NIRS technique in the study of territorial marking signals in pellets, this being a non-invasive technique which does not imply the capture or manipulation of individuals. The rabbit is a suitable model owing to its rigid linear hierarchy and territoriality, both of which have been described since the 1950s (Mykytowycz, 1958, Mykytowycz, 1964, Mykytowycz and Gambale, 1965). Olfactory communication through scent marking via glandular secretions plays an important role in territorial marking (Ralls, 1971, Doty, 1986) and a better knowledge of territoriality may be of great use in improving the success of restocking (Moreno et al., 2004, Letty et al., 2008). The rabbit's territorial marking is particularly associated with its anal glands (Mykytowycz, 1964): the faecal pellets are coated with their secretions (Mykytowycz and Gambale, 1969) and are deposited in latrines (Sneddon, 1991), which serve to delimit territories (Mykytowycz et al., 1976). Rabbits also produce isolated pellets, which neither serve the purpose of territorial marking, nor tend to accumulate in latrines (Iborra and Lumaret, 1997).

The aforementioned latrines delimit social territories and tend to indicate the existence of established breeding groups (Myers and Poole, 1959). Nonetheless, in recently established populations (corresponding to rabbit restocking actions or newly established colonies in growing populations), the discrimination between latrines and

isolated pellets is not so straightforward. Few small pellet groups are found in these situations, and latrines are operationally defined as any accumulation of 20 or more pellets in an area of 20x30 cm^2 (Virgós et al., 2003). This definition permits the comparison of rabbit abundance estimates among different studies (Virgós et al., 2003, Calvete et al., 2006, Barrio et al., 2010a, Fernández-de-Simón et al., 2011). However, the social communication function of latrines (Mykytowycz, 1964) should also be tested for a better understanding of the territorial marking process. Previous research (Letty et al., 2008) and non published data point out that the release of wild rabbits in territories inhabited by resident social groups can lead to social disruption (McKittrick et al., 2009) resulting from agonistic interactions (King, 1973). Stable rabbit populations have probably got through the initial phase characterized by high mortality levels related to physiological stress (Dickens et al., 2010), habitat novelty and predation (Calvete et al., 1997, Rouco et al., 2008), and their probability of survival and success if undisturbed is thus higher than that of a newly released group, and it would therefore be advisable to develop a fast and objective tool to identify a structured rabbit population when restocking rabbits (Mykytowycz, 1958), signifying that counterproductive actions could be avoided.

In order to identify populations with established hierarchies and territorial organisation (Mykytowycz, 1958), it would be useful to be able to detect territorial marking activity from the earliest stages. Unfortunately, the complexity of the chemical composition of anal secretions (Goodrich and Mykytowycz, 1972) -up to 56 volatile components of behavioural significance detected in rabbit pellets (Goodrich et al., 1981)- and the lack of knowledge with regard to the fractions which contain the territorial information (Alberts, 1992) make the identification of a chemical hallmark with which to define pellets with a territorial marking function difficult, if not

impossible. On the other hand, the NIRS technique has a low sensitivity in comparison to other analytical techniques such as gas chromatography or mass spectrometry (Burns and Ciurczak, 2001), and it has difficulties in detecting components at ng/g faeces concentrations. Low-concentration components would contribute little if any to the overall spectra, and the combination of overtones in the near-infrared range (Burns and Ciurczak, 2001) would prevent clear relationships between the spectral profile and chemical components from anal glands from being found. Regretfully, the large size and physical heterogeneity of the particles in the samples does not allow their analysis in the middle-infrared range (Pasikatan et al., 2001), which is the range in which the major absorption bands are to be found. Nonetheless, the combination of all the low-concentration components led us to expect differences in the near-infrared absorption profiles of marked *vs* unmarked pellets, owing to the bias in the production of pellets - adult dominant males are the main latrine producers and anal secretors (Mykytowycz, 1964, Mykytowycz, 1966, Hesterman and Mykytowycz, 1968, Mykytowycz and Hesterman, 1970, Sneddon, 1991)- and the vibration of the chemical bonds of anal secretions in near infrared frequencies (Ciurczak, 2001). Moreover, alternative theories regarding olfactory reception mechanisms (Turin, 1996) support the use of the spectrometer as the most effective chemical analysis method by which to study odours.

The aim of this study is to test the viability of NIRS as an objective, easy and fast technique for the detection of territorial marking in rabbit pellets, whilst quantifying and correcting the main sources of error. Thus, in a first model sample processing method and analysis steps will be optimized, and in a second model the effect of phenology will be minimized, thus reducing variability in both diet and the territorial marking in pellets.

Materials and methods

Study area and rabbit populations

The first group of samples was collected in central Sierra Morena, Córdoba, Southern Spain (38º 5' N, 5º 16' W). The main ecosystems in this study area include Mediterranean scrubland, pine forest and oak savanna (dehesa), which currently contain low-density rabbit populations. The samples were collected inside fourteen rabbit resettlement enclosures of 1.21 ± 0.14 (SE) ha in which rabbits were released in September 2008. All the fences were built to exclude terrestrial predators, and water and food (a grain and rabbit feed mixture) were supplied *ad libitum* during the entire study period. The rabbits were captured and randomly released inside the enclosures on the same day, within the natural distribution area of the subspecies *Oryctolagus cuniculus algirus* (Branco et al., 2000) and in the same sex-ratio as in capture, and they were not subsequently submitted to any kind of artificial selection.

The second group of samples was collected in cereal and olive fields in the south of Córdoba (37º 34' N, 4º 37' W), from which the rabbits restocked in the aforementioned enclosures had been captured. The climate is dry Mediterranean in an agricultural landscape. This area has medium to high rabbit densities, and small-game hunting is an important activity.

Sample selection

According to the main objectives, the first group of samples was collected in order to determine the effect of the processing methods: 53 samples of fresh pellets were taken between February and November 2009, 27 from latrines and 26 consisting of isolated pellets. Each sample was made up of between 50 and 100 fresh pellets and we avoided collecting excessively wet, dry or urine-contaminated pellets (Tolleson et al., 2007). These requirements prevented us from collecting pellets on days on which it

was raining or the temperature was high. After being collected, each sample was divided into 5 sub-samples, to which a different process was randomly assigned.

The collection of the second group of samples was designed with the intention of minimizing their chemical variability, which is principally caused by variations in diet in the marked territory. This was done by taking all the samples throughout the month of May 2010, during the reproductive peak, from the same agricultural area containing homogenous vegetation. Eighty seven pellet samples were used to develop the discriminant equation, 37 of which were isolated pellets and 50 of which were latrine pellets. Each sample consisted of between 10 and 15 fresh pellets, in accordance with the minimum necessary volume for the NIRS analysis. In the first two weeks of June, 38 more samples were collected in the same area in order to carry out the external validation of the discriminant equation, 22 of which were isolated pellets and 16 of which were latrine pellets. These samples were kept in a fridge at 4ºC and were then analysed between 2 and 4 hours after their collection.

The latrines from which samples were collected were selected on the basis of their large size and distinctive structure. We considered the definition of latrine provided by Virgós *et al* (2003), which is supported by the experimental observation that 20 rabbit pellets are sufficient to provide territorial responses for rabbits (Mykytowycz et al., 1976), but in order to avoid misidentifications, given that we were looking for latrines that were as unmistakable as possible for the development of the model, and considering the actual subjectivity in the practice of latrine identification, as an additional precaution, the accumulation of fecal pellets' in latrines was considered in relation to the pellets' density in the study area.

The reliability of the qualitative classification of samples in isolated or latrine pellets was calculated as the measure described by Perreault and Leigh (Perreault and

Leigh, 1989), starting with 30 samples which were classified by 5 experienced independent observers into two categories -isolated or latrine pellets. The classification made by the majority of the observers was considered to be correct. The measure described by Perreault and Leigh estimated the percentage of agreement among the observers as being 92.7%, and the classifications can thus be considered to be consistent and not subject to chance, therefore allowing us to carry out the following analyses.

Sample processing methods

The sample processing method may affect the conservation of both the marking signal and the excrement matrix. The preparation of faecal samples for the NIRS analyses shown in previous works has been carried out through the collection of fresh samples and their subsequent drying with a heater at a low temperature, 60-65ºC (Dixon and Coates, 2009). However, the drying process may impair the measurement of volatile components, which are highly important in olfactory signals (Goodrich et al., 1981, Salamon and Davies, 1998, Zhang et al., 2002). Furthermore, in the first works in which the anal gland secretions of the wild rabbit were analysed, a drying process was not used, and the whole glands were stored in ice (Goodrich and Mykytowycz, 1972), at a temperature of -27º C (Hesterman et al., 1976), or were freshly homogenised (Goodrich et al., 1978). Previous experiments have also indicated that frozen samples continue to provoke a response from the rabbits, which suggests that freezing does not eliminate the chemical signals in pellets (Hesterman et al., 1981).

In this study we compared the various processes habitually used to prepare samples. Each sample was divided into 5 sub-samples, to which a different process was randomly assigned: the first sub-samples were analysed without having been processed (Goodrich et al., 1978) after being refrigerated at 4ºC for a maximum of 48 hours. The second sub-samples were frozen at a temperature of -20ºC less than 24 hours after their

collection in order to determine the effect of freezing (Hesterman et al., 1976). A third group of sub-samples was frozen at -80ºC, and was then freeze-dried (Gidenne, 1992). The effect of the homogenisation process on the samples was studied by grinding other frozen sub-samples and maintaining them in an open envelope for an hour before the analysis. Finally, subsamples were left to dry at ambient temperature for 7 days in order to determine the utility of non-fresh pellets, which are far more abundant in the country, and to ensure by comparison that the samples were collected freshly.

Sample presentation and NIR spectral analysis

Prior to the NIRS analysis, each subsample was ground using a horizontal grinding mill. The ground samples' reflectance spectra (μlog(1/R)) were measured from 400 to 2498 nm every 2 nm, using a quartz ring cup with a diameter of 3.8 cm in a Foss-NIRSystems model 6500 (Foss-NIR Systems Inc., Silver Spring, MD, USA) equipped with a spinning module. Two spectra from each sample were collected and averaged with WinISI software v1.5 (Infrasoft International, Port Matilda, PA, USA).

The reflectance measurement repeatability, as defined by Shenk and Westerhaus (Shenk and Westerhaus, 1995), was calculated as the quadratic mean from the two spectra of each sample, showing the average distance between their reflectance values throughout the whole spectra.

Statistical analysis

The statistical analysis of the spectral data was accomplished using the WinISI v1.5 software package, following the methodology for the development of qualitative models described in the bibliography (Shenk and Westerhaus, 1995, Shenk and Westerhaus, 1996, Mark, 2001, Workman Jr., 2001). The Partial Least Squares (PLS) method was used to compress multidimensional data and reduce inter-correlated variates -wavelengths- to as many as ten independent factors. The PLS discriminant

analysis maximized the separation between the latrine and isolated sample groups and provided information about the variables -wavelength nm- that carried the information to separate the two groups. Full cross-validation was used in the discriminant analyses owing to the small sample size, and the latrine and isolate pellets' error contributions were compared. Scatter effect (Pasikatan et al., 2001), related to particle size and distribution, was corrected by using the Standard Normal Variate (SNV) as a spectral correction algorithm, Detrending (DT) for baseline correction, and first and second derivatives as smoothing (Norris and Williams, 1984, Barnes et al., 1989). These derivatives also reduced the quadratic mean error of the spectra by between 6 and 23 times, thus minimizing this error to between 270 and 400 μlog(1/R). The pre-treatment which contributed to the discriminant analysis with the greatest percentage of correctly classified samples was selected for each sample processing method.

The preliminary analyses were carried out on the basis of the complete spectra. We then attempted to reduce the total error by means of wavelength range optimisation (Spiegelman et al., 1998, Skibsted et al., 2004), and other discriminant analyses were therefore carried out in the same manner for each range of 100 nm of wavelength to determine the processing method's effect on the spectra's most dicriminant zones. Differences among processing methods were analysed by using analysis of variance (ANOVA), since we found no significant departures from normality, on the basis of the percentage of correctly classified samples resulting from the discriminant analyses carried out in each 100 nm range.

Results

Latrine vs. isolated pellets' discriminant analysis and comparison of sample processing methods.

Upon considering the discriminant analysis results from the whole spectra, the least successful results of the five groups presented were observed in the old-dried samples (Table 1), whose discriminant analysis led to results of less than 50%. We then carried out a one-way ANOVA test on the results of the discriminant analyses in intervals of 100 nm (Figure 5), and this indicated significant differences between the processing groups (d.f.=4; $p<0.001$): the *post hoc* analysis specified the old-dried sample group as that which significantly differed from the other groups (Tukey: $p<0.05$). As will be observed in Figure 5, the 900 to 1200 nm range maximized the separation between the old-dried samples and the other processing groups, and the range from 2100 to 2500 nm also showed poorer discrimination between old-dried latrine and isolated pellet samples.

Table 1. The results of the discriminant analyses for the first sample group: latrine vs. isolated pellets' discriminant analyses' results are shown corrected by the optimum mathematical pre-treatments, considering the full spectra –from 400 to 2500 nm- and applying a full cross validation. The five sample processing methods are compared: non-processed, frozen, freeze-dried, homogenised and old-dried. The percentages of correctly classified samples are shown for all the samples, the isolated samples alone and the latrine samples alone.

Sample	No. of spectra			Mathematical	% correct classification		
processing method	total	isolated	latrine	pretreatment	total	isolated	latrine
Fresh	53	26	27	SNV&DT, 2nd derivatives	60.38	66.67	53.85
Frozen	53	26	27	SNV&DT, no derivatives	60.38	61.54	59.26
Freeze-dried	53	26	27	SNV&DT, 1st derivative	58.49	57.69	59.26
Homogenised	53	26	27	SNV&DT, no derivatives	60.38	61.54	59.26
Old-dried	53	26	27	SNV&DT, 1st derivative	49.06	46.15	51.85

The discriminant analyses of the other groups correctly classified around 60% of the samples (Table 1), the highest percentage of correctly classified samples being achieved by the spectra of the freeze-dried samples in the range of 700 to 800 nm (64.15%, Figure 5). The group of fresh samples showed the greatest differences between latrine -53.85- and isolated samples' -66.67- percentages of correct

classifications on full spectra (Table 1). The different processing groups -non-processed, frozen, freeze-dried and homogenised- did not consistently show major differences across the 100 nm ranges of the spectra (Figure 5), but varied remarkably in their most discriminating ranges, the percentage of correctly classified samples depending not only on the processing method and the classification -latrine or isolated pellet- of the sample, but also on the wavelength range.

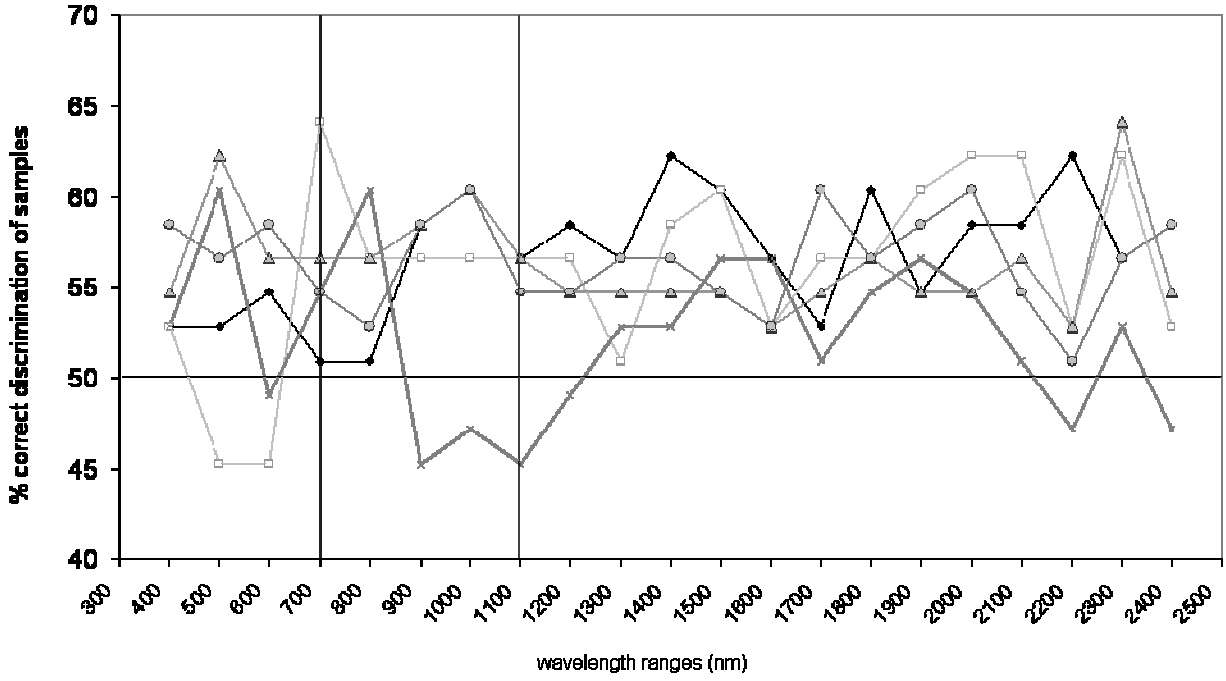

Figure 5. The percentages of correctly classified samples that resulted from discriminant analyses are plotted against the light spectra from 400 to 2500 nm, in 100 nm wavelength ranges. The sample processing methods are compared with the optimal mathematical pre-treatment chosen for each sample group: non-processed (SNV and DT and 2nd derivatives) —◆—; freeze-dried (SNV and DT and 1st derivative) —□—; frozen (SNV and DT and no derivatives) —○—; homogenized (SNV and DT and no derivatives) —△—; old-dried (SNV and DT and 1st derivative) ——. The light spectra in the x-axis is divided into three major ranges: the visible spectra from 400 to 700 nm; near-near infrared spectra from 700 to 1100 nm and near infrared spectra from 1100 to 2500 nm.

Bearing in mind the 7.3% error that resulted from the test used to measure the reliability of the qualitative classification of samples (see Materials and methods), these results would explain up to 71.45% of the variability observed.

Phenology effect and optimisation of the discriminant model.

In order to minimize the samples' chemical variability, new discriminant analyses were carried out by using a set of 87 samples collected in one sampling spot in May 2010. A different set of 38 samples was then used for the external validation (Table 2). Of the five processing methods studied above, we consider the most successful calibrations to be those obtained from the spectra of the non processed samples corrected with SNV, DT and 2nd derivatives, and these new samples were accordingly analysed less than 5 hours after collection and their spectra corrected by the aforementioned mathematical pre-treatments.

Table 2. The results of the discriminant analyses for the second sample group: latrine vs. isolated pellets' discriminant analyses' results are shown, corrected by the optimum mathematical pre-treatment, considering the full spectra –from 400 to 2500 nm- and applying a full cross validation and the external validation of the discriminant equation. The percentages of correctly classified samples are shown for all the samples, the isolated samples alone and the latrine samples alone.

Sample	No. of spectra			Mathematical	validation	% correct discrimination		
group	total	isolated	latrine	pretreatment		total	isolated	latrine
calibration	87	37	50	SNV&DT, 2nd derivatives	full CV	85.10	81.08	88.00
validation	38	22	16	SNV&DT, 2nd derivatives	external	73.68	63.64	87.50

The discriminant equation developed from the full spectra -400 to 2500 nm- and SNV, DT and second derivatives as a mathematical pre-treatment classified 85.1% of total samples correctly in the full cross-validation (Table 2): 88% of latrine samples were correctly classified, in sharp contrast to the 59% achieved with the previous and more broadly sampled group (Table 1). This 88%, when added to the 7.3% error in the

qualitative classification of samples (see Materials and methods), would explain 95.3% of the variability observed: possibly less than 5% error was attributable to the discriminant analyses of NIR spectra.

The resulting discriminant equation was then used to predict group membership for another 38 samples in the external validation. The mean percentage of correct determinations was 73.68: 87.5% of the latrine samples, and 63.64% of the isolated pellet samples were correctly predicted (Table 2).

Discussion

Our results suggest that the faecal near infrared reflectance spectroscopy is a feasible method with which to detect chemical territorial marking signals in rabbit pellets. In the first part of the study, discriminant analyses were carried out by using samples collected from different sample spots throughout the year. In the second part, all the samples were collected in the same month, coinciding with the reproductive peak, and in a different area of homogeneous vegetation, thus allowing us to minimize the chemical variation of the samples associated with phenology and diet. The secretion of the anal glands increases during the reproductive period (Hesterman and Mykytowycz, 1968), which is conditioned by the availability of food (Wood, 1980) and, owing to the rabbits' social structure (Mykytowycz, 1958), the defence of territory by the dominant male and female also intensifies, since the possession of a burrow greatly determines their reproductive success (Mykytowycz, 1959b, Mykytowycz, 1959a). Both motives led us to expect a greater difference between the isolated and latrine pellets during this period, and, with the aim of obtaining a better and more useful model in mind, we therefore collected a new set of samples during which the collection period was restricted to the breeding season. Moreover, upon limiting the samples to a single

month and to an area of homogenous vegetation, the variety in diet was reduced, which is one of the principal sources of variation in the NIRS faecal analyses (Dixon and Coates, 2009). As a result of the reduction in diet variability and the intensification of territorial behaviour during the breeding season, the percentage of correctly classified samples increased from 60% to 85%. Furthermore, 88% of the latrine samples were correctly classified, despite the fact that we ignored the sex, age and social status of the producers of latrine pellets. The discrimination was less accurate for isolated pellet samples, in both the cross and the external validations. These results suggest that, during the reproductive peak, those individuals that mark their territory through the use of latrines could also mark their territory with isolated pellets, a suggestion worth considering when the technique is applied to the detection of social structures. However, in the discriminant analyses carried out with the samples collected throughout the annual cycle, the latrine pellets showed higher error, which could be a result of the variability in marking according to the time of year (Hesterman and Mykytowycz, 1968, Mykytowycz and Hesterman, 1970).

In addition, the first model was used to study the error from sample processing. Our results agree with previous studies (Hesterman et al., 1976, Hesterman et al., 1981) in which freezing did not have a negative effect on the marking substances in the pellets, as was also the case with freeze-drying and homogenisation. Old-drying, however, did have a significantly negative effect on the detection of territorial marking, suggesting some loss of information in the process. Previous studies indicate that rabbits discriminate between fresh and dry pellets (Schalken, 1976). This loss of marking with the age of the pellets could be related to the volatile nature of certain marking components (Goodrich et al., 1981), which would explain the need for continual renewal, and which concurs with observations made in relation to the great frequency

with which rabbits visit their latrines and deposit new pellets (Mykytowycz and Gambale, 1969, Mykytowycz and Hesterman, 1970, Sneddon, 1991). In accordance with this result, we reduced the time that elapsed between the sample collection and its NIRS analysis to less than 4 hours in the second model, since the drying process might impair the measurement of volatile components, which is very important in olfactory signals (Goodrich et al., 1981, Salamon and Davies, 1998, Zhang et al., 2002). Moreover, the slight effect of freeze-drying on the discrimination of the samples suggests that this discrimination is not based on differences in humidity content. However, the humidity of the samples may affect the grinding process and the subsequent packing density, thus varying the diffuse reflectance intensity and diminishing the repeatability of the analysis. Spectral data pre-treatments increased the repeatability values of the analysis, thus minimizing the sample presentation effect, and linearizing the data and correcting for scattering effects (Pasikatan et al., 2001). As expected, the pre-treatments improved discrimination (Evans et al., 1993), which suggests that the discrimination was not based on physical differences between the samples.

The spectral noise resulting from scatter affects all the wavelengths, leading to a multi-collineality between them, and this masks the informative correlations as a result of the absorption of these same chemical links at different wavelengths, thus making both discrimination and its interpretation difficult (Osborne et al., 1993). The elimination of this multi-collineality by mathematical pre-treatments allowed a more accurate comparison of different ranges of 100 nm and, according to what we have observed in our results, the manner in which the samples are processed affects the wavelength ranges that better discriminate between samples. This could be related to the

effect of each process on the different components, whose overtones and combination bands contribute to the discrimination in different ways (Workman, 2001).

In conclusion, our results suggest that the F.NIRS is an easy to use, fast, cheap, and non-invasive tool which is able to detect territorial marking by the European rabbit. This finding suggests that the method could also be useful in the study of other complex chemical compounds even if the single components' concentrations were below the NIRS detection threshold. The results also show that these signals are lost after a few days, and that the way in which the sample is processed affects the most informative absorption bands. The results may point to phenology as one of the principal sources of error in the detection of marking signals in pellets, which probably affects both the chemical composition of the pellets and the intensity of the marking signals.

CHAPTER 2

Faecal markers for the identification of European rabbit (*Oryctolagus cuniculus* L.) social territories.

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Introduction

The definition of a spatial framework is essential to ecological studies (Kneitel and Chase, 2004), but its scale depends on the objective: habitat, home range, management units or territory are some of the spatial scales on which studies concerning diet, species distribution or social behaviour have been based (Mykytowycz et al., 1976, Barrio et al., 2010b, Carter et al., 2012, Massé and Côté, 2012).

Territorial units (Maher and Lott, 1995) become important when studying social species (Jordan et al., 2007). Territories vary according to the availability of food, space, population density or habitat, since the intensity of territorial behaviour is conditioned by the trade-off between the cost of defending a territory and the benefits obtained from resource monopolisation (Maher and Lott, 2000). This variability makes it difficult to define spatial frameworks that are based on social behaviour.

Our case study was the European rabbit (*Oryctolagus cuniculus* L.), a species that is subject to intense and diverse management efforts: it is an endangered keystone

species in its native range, the Iberian Peninsula, while it is an invasive pest in the range into which it has been introduced worldwide (Delibes-Mateos et al., 2011). Both restocking (Letty et al., 2008) and pest control programs (Smith et al., 2007) are therefore being simultaneously applied in different parts of the world. For both kinds of efforts, more in-depth studies concerning rabbits' social territories might be helpful, particularly because territoriality and social structure are two major aspects of rabbit biology (Mykytowycz, 1964).

Rabbits delimit their social territories with latrines, which tend to indicate the existence of established breeding groups (Myers and Poole, 1959), and territoriality in rabbits has therefore been studied via latrine distribution by direct visual observation (Mykytowycz and Gambale, 1969) or latrine mapping (Monclús and De Miguel, 2003). Nevertheless, the results of these studies are limited by the laboriousness of the mapping and the difficulty of observing rabbits in the wild. Moreover, the resolution in the identification of latrines as belonging to a social group is lower the further the faeces are deposited from the original burrow entrances.

In this work we have identified latrine nets belonging to the same social group through the use of indigestible faecal markers. Food colorants and other markers are widely used in zoological institutions (Fuller et al., 2010) with the aim of discriminating between different individuals' faeces for analytical purposes, but marked baits have also been fed to wild badgers in order to improve the study of their territorial boundaries and animal density (Delahay et al., 2000). The bait-marking method can provide fundamental information on the configuration of social groups and create a spatial framework for ecological studies. It requires no specialist equipment, the results can be relatively easy to interpret, and the location of territorial boundaries is far less labour intensive than radio-tracking or direct observation (Delahay et al., 2000).

The aim of this work is to test the feasibility of non-digestible faecal markers in the identification of the territories of wild rabbit social groups.

Materials and methods

In order to choose the best means of feeding indigestible markers, different kinds of baits and faecal markers were tested on wild rabbits in captivity and semi-captivity. Captive and semi-captive rabbits were located in 6 rabbit enclosures situated in a Mediterranean scrubland in central Sierra Morena, Córdoba, southern Spain -38º 5'N, 5º 16'W. The captive rabbits were kept in two 0.5 ha enclosures during March and April 2011, while the semi-captive rabbits had been kept in 4 enclosures of 4.5 ha since October 2010. Fenced plots were built to exclude ungulates and terrestrial predators, and artificial warrens were built within the plots, 50 to 100 m apart from each other, to promote rabbit reproduction. Food and water were supplied *ad libitum* throughout the entire year.

The baits were chosen on the basis of their palatability and capacity to fix the markers: bread, cereal grains and vegetable mixture were provided *ad libitum*, coated with different markers: red, yellow and blue water-soluble food colouring (Vahiné, McCormick, Portugal); red, yellow and blue lake colours (Roha Europe, S.L.U., Torrente, Spain); and non-toxic pearly-coloured (different pearly colours) and phosphorescent glitter (Martha Stewart Crafts, Sulyn Ind., Coral Springs, Florida, USA). Fresh pellets were observed 12-36 hours (Monclús et al., 2006a) after each marker had been supplied.

The captive rabbits had a one week acclimation period inside the enclosures until all the palatable vegetation cover had been eaten; they were then fed with the different bait-marker combinations, with 48 hours on non-marked food between

successive tests. The successful baits and markers were then fed to the rabbits inside the 4 large enclosures during the reproductive peak, in May 2011, when territorial defence is most intense (Mykytowycz, 1959a, Hesterman and Mykytowycz, 1968). Three different markers were used in 3 adjacent artificial warrens per enclosure in order to mark latrines belonging to different warrens with different colours. The warrens were sufficiently far apart to guarantee that they belonged to different social groups, and the presence of fresh latrines in all burrow entrances suggested active territorial behaviour. New marked baits were put out weekly, for 3 weeks, in the burrow entrances to ensure that they would only be available to the rabbits belonging to those warrens. In the fourth week we geopositioned (GPS Zeno 10, Leica, with IP67) the latrines in the area surrounding the warrens, limited by the fences and the neighbouring warrens, noting all the marked pellets. We considered a latrine to be any accumulation of 20 or more pellets in an area of 20x30 cm^2 (Virgós et al., 2003), and we considered any latrine that contained at least one marked pellet to be marked.

The spatial distribution of marked latrines was analysed in relation to the position of the warrens and the food and water supply points. The latrine-marked areas were measured using Minimun Convex Polygons (MCP). The differences in intensity of the territorial marking throughout the latrine-marking areas were studied using Kernel density estimators. The smoothing factors were calculated using least-square cross-validation (Worton, 1989), and 50% isopleths were used to define maximum latrine density areas inside the kernels. These areas were considered to be the nucleus zones in which the rabbits were particularly active in defence of their territory. The spatial data were analysed using ArcGIS 9.3 and fixed kernels and MCP were created with the extension Hawth Tools for ArcGIS (Beyer, 2004).

The study was approved by the University of Córdoba's Ethical Committee for Animal Experimentation.

Results

When tested on captive rabbits (Table 3), none of the water-soluble colourings marked the faeces. Blue lake colouring clearly marked the faeces, but yellow and red did not. The glitter was detectable in the faeces in a low concentration, but magnifying glasses were required to discriminate the colour of the particles, and direct visual detection was more difficult than with the blue lake-marked pellets. All three baits were successful, particularly the bread owing to its porosity, which allowed the lake and glitter particles to adhere well. On the other hand, the semi-captive rabbits' food supply was not controlled: vegetable mixtures were not eaten, and cereal grains were stolen by ants in a few minutes, even those inside plastic containers. The bread was successful inside the enclosures (Table 3) and this was therefore the substrate chosen to carry out the experiment.

Marked pellets were found in the latrines of 3 of the semi-captive rabbit enclosures. The majority of marked pellets were marked by blue lake colouring, but we also identified a few glitter-marked pellets. However, slate and dried clay caused the ground to shine, thus making the identification of glitter difficult, and these data were therefore excluded from the analyses. The measures of the latrine-marked areas, calculated using MCP, are summarised in Table 4. In Figure 6, maximum latrine-marking activity zones can be observed, estimated using the kernels' 50% isopleths.

Table 3. Summary of successful ("yes"), unsuccessful ("no") and feasible but not useful ("marginally") markers and baits tested on wild rabbits in conditions of captivity and semi-captivity.

Environment			Captivity	Semi-captivity
Marker	water-soluble colouring	yellow	no	-
		red	no	-
		blue	no	-
	lake colouring	yellow	no	-
		red	no	-
		blue	yes	yes
	non-toxic glitter	pearly colours	yes	marginally
		phosphorescent	no	no
Bait	Bread		yes	yes
	cereal grain		yes	no
	vegetable mixture		yes	no

Table 4. Estimation of latrine marking areas, perimeters and warren-to-latrine distances (maximum distance and average ± standard deviation) of 3 territories in 3 rabbit restocking enclosures, according to Minimum Convex Polygons (MCP) analyses of 100% of blue lake marked latrines. Marked latrine number per enclosure (N) is provided.

		Latrine-warren distance (m)		MCP	
Enclosure	N	maximum	average ± SD	area (m2)	perimeter (m)
A	17	29.7	12.1 ± 8.7	562.6	104.0
B	8	39.3	25.3 ± 8.1	323.0	88.2
C	14	24.2	13.4 ± 5.7	703.5	108.7

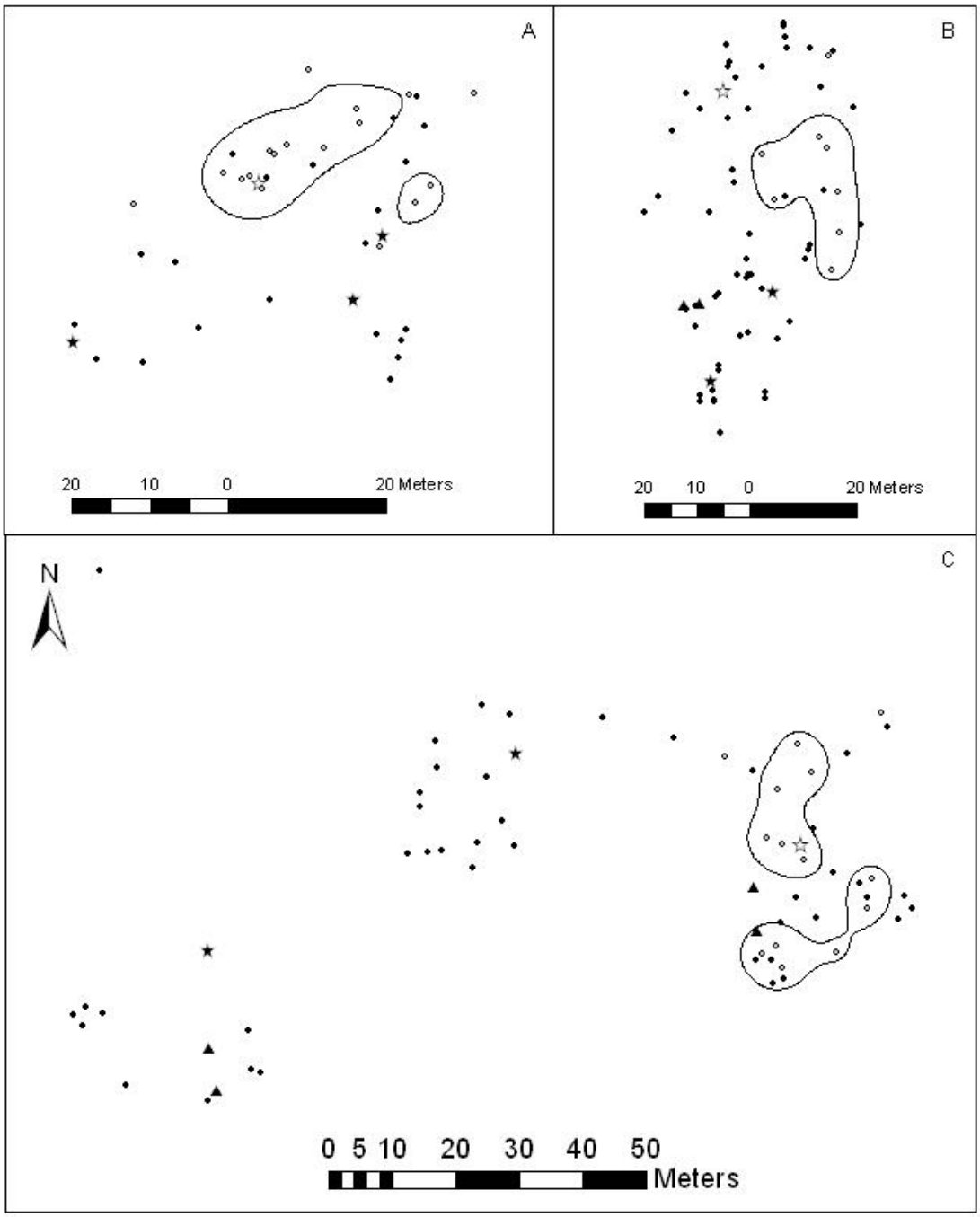

Figure 6. Kernel 50% isopleths showing maximum marking activity areas in 3 rabbit restocking enclosures. The blue lake-marked latrines are represented as empty circles, and the unmarked latrines as black circles. Empty stars represent the warrens which were supplied with blue lake-marked bait, while black stars represent the warrens supplied with the other unsuccessful markers. Black triangles represent the enclosures' food and water supply points.

Discussion

Of all the bait-marker combinations tested, the bread with blue lake colorant was the only one to mark latrine nets in a detectable manner. Glitter particles were also detectable in captive rabbits, but not under semi-natural conditions, and water-soluble colorants could not be observed in any faeces, perhaps as a result of rabbits' double digestion system (Southern, 1940), although no marked soft faeces were found either.

In summary, the use of faecal markers allowed the visual detection of latrines recently used by individuals belonging to identified warrens. The blue lake colorant permitted the detection of latrines belonging to identified warrens and, then permitted the delimitation of the territory of the social groups of those warrens. We identified 3 territories in 3 large enclosures which shared similar characteristics as regards habitat and management, but in which all the latrine-marking areas differed in size, shape and nucleus position relative to the warrens. The nuclei of the marking areas corresponded, respectively, to the warren, the between-warren area and the feeding point, suggesting different solutions to the aforementioned cost-benefit trade-off.

Monclús and De Miguel (2003) observed an average latrine-to-warren distance of 16 m and a maximum distance of 60 m in free rabbits from a similar environment. The average distance inside our enclosures ranged from 12 to 25 m, and the furthest latrines were located at 24 to 39 m from the warrens, the latrine-marking areas ranging from 323 to 703 m^2. These distances resulted from two aspects of the land use by wild rabbits: the spatial aggregation of rabbit burrows (Barrio et al., 2009) and territoriality during the reproductive season (Mykytowycz, 1959b, Mykytowycz, 1959a, Hesterman and Mykytowycz, 1968). Rabbits are gregarious and tend to aggregate despite hostilities (Mykytowycz and Gambale, 1965): females tend to cluster on the best breeding sites and the males aggregate to defend this resource - females - until the expansion of the

colony is limited by social interferences (Cowan, 1987). The artificial warrens may therefore become the territory of the dominant pair, while the subordinate individuals are excluded (Mykytowycz, 1958)**.** The integrity of the colonies is partly maintained by latrines (Mykytowycz and Goodrich, 1974) by permeating the territory and enhancing territorial confidence (Mykytowycz et al., 1976), and by furthermore separating social groups by concentrating between adjacent warren systems (Mykytowycz and Gambale, 1969). These behavioural traits led us to expect two nuclei in latrine distribution: the first in the artificial warrens, protecting the main resource during the reproductive season - nest sites -, and the second defining the boundaries between adjacent warrens. The kernel areas identified these two zones in different territories, reflecting different interests in different groups. The kernel for enclosure C identified another nucleus: the artificial feeding site. This nucleus was secondary to the warren, but very interesting, as it indicated that food is a limiting resource inside the enclosures.

In conclusion, the faecal markers coloured wild rabbit social territories under semi-natural conditions, and were proved to be a useful tool by which to explore the territorial behaviour of wild rabbits, in addition to providing easy clues with which to define spatial frameworks based on social structures.

CHAPTER 3

Acute stress reactivity in European rabbit (*Oryctolagus cuniculus* L.): Effect on productivity

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Introduction

Stress is a physiological and behavioural reaction to the perception of stressors, which may be caused by predation, environmental or anthropogenic disturbance, weather or social disruption (Wingfield et al., 1998). The brain responds to this perception by activating a cascade of hormones in the hypothalamo-pituitary-adrenal (HPA) axis, ultimately leading to the secretion of glucocorticoids (GCs) which lead to physiological and behavioural changes whose objective is to cope with the stressors, recover the homeostasis and enhance survival (de Kloet et al., 2005): this is the so-called acute stress response, a rapid elevation of GCs that induces responses to life-threatening situations and is then quickly cleared from the organism. Acute stress response is assumed to be adaptive in nature (Breuner et al., 2008), but when sustained elevations of GCs occur the stress becomes chronic. The physiological cost of maintaining the homeostasis in these conditions is high: immunosuppression (Moynihan and Ader, 1996), impaired cognitive functions (de Kloet et al., 2005), illnesses related

to the digestive system (Taché et al., 2001), loss of body mass (Cabezas et al., 2007) or impaired reproductive performance (Von Holst, 1998). Stress is therefore considered to play an important role in animal management programs (Dickens et al., 2010) owing to the GC-fitness relationship (Bonier et al., 2009) and the adverse effects of chronic exposure to stressors explained above. However, despite the known negative effects of chronic stress, optimal acute stress reactions continue to be unknown (Breuner et al., 2008).

Our case study was the European rabbit (*Oryctolagus cuniculus* L.), a native species in the Iberian Peninsula (López-Martínez, 2008b), which plays an important role as a key prey species in Mediterranean ecosystems (Delibes-Mateos et al., 2007). Rabbit populations have undergone a dramatic decline in abundance in their natural ranges (Virgós et al., 2003) which has principally been caused by viral diseases (Moreno et al., 2007, Williams et al., 2007) and a loss of suitable habitat (Calvete et al., 2004a). Rabbits are therefore currently being subjected to many conservation and restocking efforts in order to improve their availability for predators (Moreno et al., 2004). The process of rabbit restocking in itself involves different sources of stress (Dickens et al., 2010), and the animals will have to cope with new stressors in the restocking area. One of the most important stressors is predation, either through predator attacks or predator perception by odour (Monclús et al., 2009), and the Iberian Peninsula is rich in predators that prey on rabbits, with more than 30 Iberian predator species (Delibes and Hiraldo, 1981), which strongly affect the success of rabbit restocking programs (Calvete et al., 1997, Letty et al., 2002, Moreno et al., 2004, Rouco et al., 2008).

To date, very few studies on rabbits have been performed to analyse the mechanisms underlying the relationships between stressors, physiological stress response, behavioural response and fitness. An increase in GC levels after predator

odour exposure was observed in laboratory conditions (Monclús et al., 2006a) but not so under semi-natural conditions (Monclús et al., 2006b), whereas basal GC levels showed a finely adjusted relationship with predator presence under natural conditions (Monclús et al., 2009). Moreover, a study of the relationship between exposure to long-term stress and fitness in wild rabbits found that moderately elevated GC levels were negatively associated with body condition, but positively associated with subsequent survival upon release (Cabezas et al., 2007). These studies included the analyses of serum glucocorticoids (Cabezas et al., 2007) and fecal glucocorticoid metabolites (Monclús et al., 2006a, Cabezas et al., 2007, Monclús et al., 2009) for baseline or chronic stress levels. Adrenocorticotrophic (ACTH) challenge tests were also performed to assess the responsiveness of the individuals' adrenocortical system (Monclús et al., 2005, Monclús et al., 2006a) in relation to predator odour and rabbits' behaviour (Rödel et al., 2006).

However, the physiological response of wild rabbits to acute stressors has not yet been studied in the field. The reactivity to stressors and the capacity to recover after the stressor has disappeared affect fitness (Romero and Wikelski, 2010), although little is known about either this relationship (Breuner et al., 2008), facilitation (Romero, 2004) or habituation (Gump and Matthews, 1999). Experiments in the field are therefore advisable, particularly since the relationship between the stress response and fitness is context dependent, and may vary according to environmental conditions (Blas et al., 2007). Additionally, in order to better ascertain the shape of this relationship, wide ranges of reactivity measures should be recorded, since the response to stress, like other homeostatic mechanisms, will most probably exert its effects in a U-shaped dose-response curve (Chrousos, 2009).

However, the monitoring of stress hormones in the wild involves certain methodological problems. The capture, handling and blood sampling of wild animals

are sources of stress in themselves and, apart from bioethical concerns, they may produce bias in the analysis of serum GCs (Sheriff et al., 2011). One alternative lies in non-invasive monitoring techniques: the analysis of faecal GC metabolites by radioimmunoassays (Cabezas et al., 2007) or enzyme immunoassays (Touma et al., 2003) permits the assessment of physiological levels through the collection of faecal samples without disturbing the animals.

The aim of this preliminary study is to test the feasibility of the analysis of faecal GC metabolites in order to detect differences in the acute stress response among restocked wild rabbit populations, and, were such differences to exist, to test the predictive value of the acute stress response as a non-invasive indicator of future population growth.

Materials and methods

Study area

Our fieldwork was carried out in central Sierra Morena, Córdoba, Southern Spain - 38º 5' N, 5º 16' W. The main ecosystems in our study area include Mediterranean scrubland, pine forest and oak savanna, which currently contain low-density rabbit populations. Data on faecal corticosterone metabolites (FCM) and rabbit abundance were recorded in a set of six predator exclusion enclosures for restocked rabbits (4.4 ± 0.9 (SE) ha) between 1 and 2.5 kms apart from each other. Aerial predation was considered to be homogeneous in the entire study area since all the enclosures included in the study were situated within an area of 4 km^2, a similar area to that described for the home range of birds of prey (López-López et al., 2006). The average flight time of birds of prey in the area was estimated as being 0.0296 (total

amount of flight time of the birds of prey / total number of observation hours), following the methodology of Redpath and Thirgood (Redpath and Thirgood, 1997).

Enclosures and wild rabbits

All the fences were built to exclude terrestrial predators, artificial warrens were built in each plot, and water and food (a grain and rabbit pellet mixture) were supplied *ad libitum* during the entire study period. The rabbits were captured and released inside the enclosures on the same day within the natural distribution area of the subspecies *Oryctolagus cuniculus algirus* (Branco et al., 2000), with no vaccination or quarantine measures and in the same sex-ratio as in capture. The animals were released inside the enclosures in November 2010 and they had an acclimation period of two months before the experiment was performed. The stressors were applied in January 2011 and rabbit abundance was estimated on a monthly basis from January to July 2011. Trap-cameras (Leaf River Model IR-5 & IR-7SS, Vibrashine, Inc.) were used for two days prior to the application of stressors and these confirmed the absence of terrestrial predators inside the enclosures. Canned sardines were used to attract predators on the basis of their effectiveness in previous efforts (unpublished).

Stressors

Three different stressors were simultaneously applied in the rabbit enclosures in order to mimic the most frequent sources of disturbance in the study area: the presence of a predator, the smell of a predator and human disturbance. The role of predator was fulfilled by a dog, which was allowed to walk and explore inside each enclosure and mark it with urine. The smell of a predator was obtained from fresh ferret faeces collected the previous day and preserved in a fresh state. Ferret faeces were placed in front of the main burrow systems and removed on the second day. Human

disturbance implied five people talking loudly and walking inside the enclosures. This choice of combined stressors was aimed to obtain a strong physiological stress response in the rabbits since, as the study area was a natural environment, it was not possible to control the level of impact on every individual rabbit. All three stressors are frequently found in the study area, signifying that the physiological stress response observed was a faithful representation of that usually found in these rabbit populations.

Sample collection

Faecal samples were collected in the burrow entrances of four to five burrow systems per enclosure ($N_{enclosures}$=5; $N_{samples}$=22), each sample consisting of 20 to 30 fresh faecal pellets. Fresh pellets were identified by their wet and dark appearance, adhesive nature, and their being soft to the touch, which differed from older pellets that usually are dry, lighter and harder (Monclús et al., 2009). The burrow systems were sufficiently far apart (50 to 100 m) to guarantee that they belonged to different social groups, and the presence of fresh latrines in all burrow entrances suggested active territorial behaviour. Prior to the application of stressors, 22 samples were collected to estimate baseline FCM levels (FCM_{t0}). The time delay of FCM excretion in rabbits has been found to range from approximately 12 to 36 hours (Monclús et al., 2006a); hence, 24 hours after the application of stressors, the second freshly deposited pellet group (N=22) was collected (FCM_{t24}). The last sample group was collected 72 hours after the application of stressors (N=22) in order to estimate the recovery levels (FCM_{t72}). The samples were stored at -80ºC and freeze-dried before the extraction of FCM.

FCM extraction and analysis

Each freeze-dried sample was homogenised using a horizontal grinding mill and 0.2 g was weighed with a precision balance. These aliquots were extracted with 5

ml methanol (80 %) for 30 minutes in a vortex (Monclús et al., 2006a). After centrifugation (10 minutes at 2500 g) a 0.5 ml aliquot of the supernatant of each sample was transferred to a new vial, oven-dried at 70ºC and stored until analysis. Faecal corticosterone metabolites (ng metabolites/g dry faeces) were analyzed by using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) developed for mice (*Mus musculus*) (Touma et al., 2004) and previously validated for wild rabbits (Monclús et al., 2006a).

Reactivity levels

The reactivity to the applied stressors was calculated by dividing the acute stress levels by the baseline levels (%):

$$\text{Reactivity} = FCM_{t24} / FCM_{t0} * 100$$

Rabbit abundance estimates and productivity

Rabbit abundance was estimated through the use of monthly pellet counts at fixed points (Fernández-de-Simón et al., 2011), and a pellet abundance index (PAI) was created through the average abundance of pellets per day and m^2 for every month and enclosure. Annual productivity (%) was estimated in each enclosure by dividing rabbit maximum abundance (July) by the minimum abundance (January):

$$\text{Productivity} = PAI_{July} / PAI_{January} * 100$$

Statistical analysis

Linear mixed models were used to check for differences between basal ($FCMt_0$), stressed ($FCMt_{24}$) and recovered ($FCMt_{72}$) FCM levels, and for differences in responsiveness between the different enclosures. The repeated samplings per warren (t_0, t_{24}, t_{72}) were accommodated by including warren (N=22) as a random factor. The fixed components used to explain the response variable were sampling time (N=3), enclosure

(N=5), and their interaction (time*enclosure). The FCM level measured in each warren and each time was the response variable. We considered the outliers (Navidi, 2008) as biologically relevant in this case, and we therefore log-transformed the response variable in order to include their contribution to variability in the final model. The addition of a variance structure which allowed for heterogeneity of variance between sampling times did not improve the model, and it was therefore excluded from the final model, and homocedasticity was assumed. The residuals of the final model did not show temporal correlations, and the Q-Q plot suggested normal distribution of the residuals, signifying that normality and independence were confirmed (Zuur et al., 2009).

Additive models with Local Weighted Regression Smoothers (LOWESS) were used to check for a relationship between acute stress responsiveness and productivity. We included the three outliers in the analyses since they represented the hyperresponsive individuals in the populations; hence, both the dependent variable (productivity) and the predictor (reactivity) were log-transformed. The models were compared for span widths of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8. The model with the lowest AIC (span = 0.2) was revealed to be inappropriate upon the diagnosis of model residual, and the model with the second lowest AIC (span = 0.8) was therefore selected as the final model. Normality, homogeneity and independence of the residuals were confirmed (Zuur et al., 2009).

Model selection was based on Akaike's Information Criterion (AIC) and Likelihood Tests. Model diagnosis was based on analyses of model residuals. Statistical analyses were performed using R 2.9 software (Team, 2008) and the *nlme* (Pinheiro and Bates, 2000) and *gam* packages (Hastie, 2006) were used to fit the models.

The study was approved by the University of Córdoba's Ethical Committee for Animal Experimentation.

Results

FCM levels varied significantly between sampling times ($FCMt_0$ = 212 ± 44 SD, $FCMt_{24}$ = 253 ± 157 SD, $FCMt_{72}$ = 200 ± 74 SD; ANOVA, F = 3.473, df = 2,34, p = 0.0424) and between enclosures (lowest levels = 169 ± 58 SD, highest levels = 326 ± 164 SD; ANOVA, F = 4.997, df = 4,17, p = 0.0076). What is more, the FCM levels in enclosures varied in a significantly different manner (ANOVA, F = 4.007, df = 8,34, p = 0.0019). Although in the final model the response variable was log-transformed to account for the outliers and heterogeneity, it must be noted that the acute stress levels measured ($FCMt_{24}$) reached 716 ng/g dry faeces.

In the additive model, the smoother for logReactivity was significant at the 5% level (F(1,2) = 7.81; p = 0.0038). The final model suggested a relatively linear relationship between logReactivity and logProductivity (span = 0.8), with the three outliers shifting the curve towards an inverted-U shaped relationship (Figure 7).

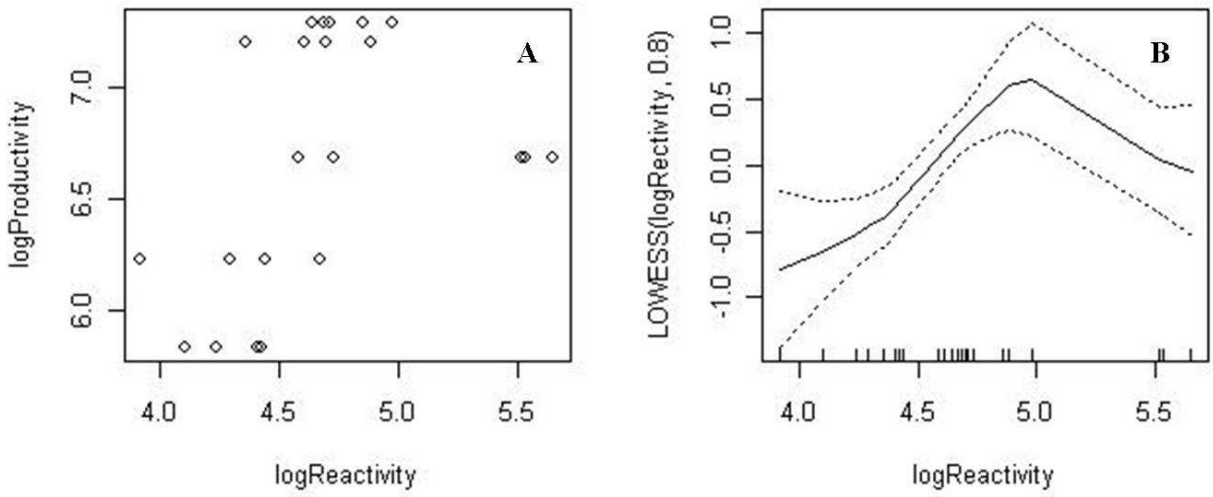

Figure 7. A: a scatterplot showing the relationship between the reactivity to acute stressors (logReactivity) and the annual productivity of our wild rabbit populations (logProductivity). B: LOWESS smoother curve estimated with span = 0.8 and pointwise 95% confidence bands obtained by the *gam* package in R.

Discussion

Few works have explored the physiological stress response in rabbits. Our initial FCM values (212 ng/g dry faeces ± 44 SD) were higher than those from other wild rabbit translocation plots in a similar habitat (86 ng/g dry faeces ± 35 SD (Monclús et al., 2009)) and were also higher than those found in rabbits bred in captivity (94 ng/g faeces ± 24 SD in males and 68 ng/g faeces ± 19 SD in females (Monclús et al., 2006a)). Furthermore, the faecal samples collected 24 hours after the application of the stressors showed FCM levels of up to 716 ng/g dry faeces, more than double the maximal capacity to secrete corticosterone obtained by Monclús e*t al* (2006a) in an ACTH challenge test. The comparison between our FCM measures and the measures obtained for in rabbits reared in captivity suggests that artificial selection processes work against hyperresponsive individuals in captivity, and hence underlines the need for more detailed research into wild animals under natural conditions.

Moreover, negative feedback is an important aspect of the HPA axis function (Dallman et al., 1994, Kruk et al., 2004). It has been described as one of the major correlations to survival probability in some species (Romero and Wikelski, 2010), and some studies show the independence of reactivity and recovery processes (Ramsay and Lewis, 2003), but the quantification of the recovery is, regretfully, usually omitted (but see (Romero and Wikelski, 2010)). Our preliminary results showed a physiological recovery 48 hours after an acute stress response in wild rabbit populations, and thus provided us with a more accurate measure of the recovery from a stress response than that described by Rouco *et al* (Rouco et al., 2011), who observed the ability to terminate a behavioural response to a stressor (the smell of predator scats) two months after the stressor was removed.

To continue with the principal aim of this study, our results did show a differential response to the stressors by the different rabbit populations, and the variability in responsiveness (or reactivity) was related to the variability in the annual productivity of the rabbit populations. The reactivity-productivity relationship was inverted-U shaped, as expected (Chrousos, 2009), with maximum productivity values in enclosures with 100 to 150 % reactivity. The outliers included in the analyses could represent hyperresponsive individuals, and we therefore regarded them as biologically relevant: hyperresponsiveness to stressors has been linked to greater mortality and a loss of fitness in different species (MacDougall-Shackleton et al., 2009), and our preliminary study suggests that, in wild rabbit populations, high levels of reactivity to stressors may be associated with lower productivity levels.

Furthermore, the consistency of the stress response inside the enclosures (the reactivity of the different social groups inside each enclosure was generally similar), after all the rabbits had been captured in the same area and released in the same sex

ratio in the enclosures two months before the experiment was performed, suggested a rapid neuroplasticity mechanism (Sousa et al., 2008) associated with environmental factors (i.e., predation, protective structures or different sources of food) which varied between enclosures, although a differential impact of the applied stimulus upon the individuals would have also been possible. Nevertheless, either way, and according to our results, the response to stressors was related to the annual productivity, and the environmental conditions that favoured reactivity levels between 100 and 150% could therefore have been related to the top productivity values.

In conclusion, the analysis of FCM is a suitable method with which to measure responsiveness to acute stressors in wild rabbits, since it was able to measure a continuous range of reactivity to acute stressors and to detect different responses by different populations. It would also be possible to relate the variability in the stress response to the variability in the wild rabbit populations' annual productivity, signifying that the predictions of wild rabbit populations' future growth could be improved by means of non-invasive measures of the acute stress response. Moreover, we wish to highlight the analyses and meta-analyses that show the changeable nature of stress responsiveness (Blas et al., 2007, Breuner et al., 2008) in order to suggest that more studies in natural conditions are required to understand the complexity of the interactions between environment, management, physiological stress responses and fitness.

CHAPTER 4

High rabbit abundance proves detrimental to productivity in European rabbit (*Oryctolagus cuniculus* L.) restocking enclosures.

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Introduction

The European rabbit (*Oryctolagus cuniculus* L.) is a native key species in the Iberian Peninsula (López-Martínez, 2008b) and is subject to many restocking actions in its natural ranges (Moreno et al., 2004) owing to the dramatic decline of its populations (Virgós et al., 2003) as a result of viral diseases (Moreno et al., 2007, Williams et al., 2007) and a loss of suitable habitat (Calvete et al., 2004a). Restocked rabbits may be produced in captive breeding programs (González-Redondo, 2003, Arenas et al., 2006) or can be translocated from localities in which rabbits are abundant (Moreno et al., 2004).

The captive breeding of wild rabbits has been carried out in intensive systems, but the process is difficult and not very productive: physical injuries (González-Redondo, 2009) and failures in maternal behaviour (González-Redondo and Zamora-Lozano, 2008) have been reported, mostly as a result of unsuitable living conditions and high levels of stress. Semi-extensive breeding systems have attained higher productivity (Arenas et al., 2006), but the lack of acclimation of the rabbits produced results in high mortality when they are released into the wild (Rouco et al., 2010).

The alternatives are extensive systems. The most frequent procedure is the translocation of captured wild rabbits to predator-exclusion fenced plots (Ferreira and Delibes-Mateos, 2010), which improves the restocking efficiency by reducing the short-term mortality rates (Calvete and Estrada, 2004) and increasing the long-term population growth (Rouco et al., 2008). Various projects, such as the Iberian Lynx LIFE project, have been responsible for building more than 260 rabbit fenced plots since 2002 (Gil-Sanchez, 2011). These fenced plots may have two aims: the establishment of supplementary feeding stations for predators (López-Bao et al., 2008) or the creation of centres for the dispersion of rabbits. In the latter case, the enclosures are breeding systems which are protected against terrestrial predators, and their aim is to increase rabbit abundance, thus enabling the rabbits to then spread and colonize the surrounding areas (Rouco et al., 2008). When enclosures are run with this aim, the maximum efficiency corresponds to the populations in the exponential growth phase (Hutchinson, 1948), that is, maximum productivity.

However, rabbit population growth inside the restocking fences is limited by predation (Calvete et al., 1997), diseases (Calvete et al., 2002) and intrinsic mechanisms, such as physiological stress and social interactions (Letty et al., 2008), since individuals compete for social rank and territory. These behavioural traits, unlike predation (Pech et al., 1992), are density-dependent (Wynne-Edwards, 1959).

In rabbit populations, fecundity and population growth decrease as density increases (Myers and Poole, 1962, Myers and Poole, 1963). More recently, Rödel *et al* (Rödel et al., 2004a, Rödel et al., 2004b) have shown that there are lower reproduction rates and a higher mortality in high rabbit densities. The aim of this work is to analyse the effect of rabbit abundance on rabbit productivity within the restocking enclosures in order to improve the productivity of extensive breeding systems.

Materials and methods

Study area

Our fieldwork was carried out in central Sierra Morena, Córdoba, Southern Spain -38º 5' N, 5º 16' W. The data on rabbit abundance included in this study originated from two sets of enclosures for restocked rabbits. The first group included 19 fences of 1.21 ± 0.14 (SE) ha in which rabbits were released in September 2008. Rabbit abundance was recorded on a monthly basis from October 2008 until July 2009, when the yearly maximum abundance was reached. The second data set originated from another 6 fences of 4.44 ± 0.93 ha, and data were recorded from November 2010 to July 2011. All the fences were built to exclude terrestrial predators, artificial warrens were built in each plot, and water and food were supplied *ad libitum* during the entire study period. The rabbits were not submitted to any kind of artificial selection. Predation by raptors within the enclosures was estimated by a census of birds of prey at 3 fixed points during springtime, with a total number of 21 hours of observation per zone (Redpath and Thirgood, 1997, Rouco, 2008). An aerial predation index (API) was then created by dividing the total amount of flight time of the birds by the observation hours.

Rabbit abundance estimates

Rabbit abundance was estimated by monthly pellet counts at fixed points (Catalán et al., 2008, Fernández-de-Simón et al., 2011). A pellet abundance index (PAI) was created through the average abundance of pellets per day and m^2 for every month and enclosure. All the statistical analyses were developed using the abundance estimates based on pellet counts. Upon considering the utility of a direct relationship between pellet-counting estimates and the actual number of rabbits in our enclosures, a linear

regression model (LR1) was then developed using non-published data concerning the number of rabbits released in nine empty enclosures, the size of the enclosures, the days spent inside them prior to the first pellet-counts, and the number of pellets counted in them. In these nine enclosures, the rabbits were released in autumn and had the same kind of food *ad libitum*. The number of rabbits released was corrected by a short-term mortality rate of 66% estimated in adjacent predator exclusion fences, similar to those estimated in other translocation fences (Cabezas et al., 2011). The number of rabbits per hectare could be calculated as (adjusted R^2=0.73; $F(1,8)$=25.4; $p<0.001$): N rabbits / ha = [1323.9 * (nº pellets/m2/day) + 141.75] / (days since the last pellet-count)

Statistical analyses

In order to account for the autumn productivity, we estimated the relative increase (%) in rabbit abundance from November to January ((PAI_{Jan}-PAI_{Nov})/PAI_{Nov}*100). We used the data from the 11 enclosures in set 1, since in the rest of set 1 rabbits had been released in November 2009, and autumn productivity might therefore have been affected by the acclimation period after release. We developed a linear regression model (LR2) in which autumn productivity was the dependent variable and the logarithm of November pellet abundance (ln PAI), the logarithm of enclosure size (lnSize) and the aerial predation index (API) were the independent continuous variables. Both the ln-trasformation of the variable Size and PAI improved normality and homocedasticity according to the analysis of model residuals (P-plot and predicted-residuals plot). Spring productivity was estimated in each enclosure as the relative increase in rabbit abundance (%) from March to July ((PAI_{July}-PAI_{March})/PAI_{March}*100). We used the data from the 16 enclosures in set 1 (it was necessary to exclude 2 enclosures owing to management problems and another because the analysis of residuals suggested it to be an outlier) and the 6 enclosures in set 2. We developed a

linear regression model (LR3) in which spring productivity was introduced as a dependent variable, the logarithm of March pellet abundance (lnPAI), the enclosure size and the aerial predation index (API) were the independent continuous variables and the data set was the independent categorical variable. The analysis of model residuals suggested normality (P-plot) and homocedasticity (predicted-residuals plot). Once non-significant variables had been excluded from the spring model, another model (LR4) was developed to check the possible effect of autumn productivity on spring productivity. Only 9 pieces of data were available for this model. Spring productivity was the dependent variable, and the independent variables were autumn productivity and lnPAI, the only variable included in the previous model (LR3). The data were analysed by Statistica and SPSS software using linear regression models, and Akaike Information Criteria corrected for small sample size (AICc) (Akaike, 1974, Burnham et al., 2011) was used to choose the best-fit models.

Results

The first model (LR2) chose the logarithm of the pellet abundance index (lnPAI) and the logarithm of enclosure size (lnSize) as the most parsimonious model to explain autumn productivity (Table 5). The variable lnPAI described 53.3% of the variability of the model and affected the autumn productivity in a negative manner (β=-0.82). lnSize additionally described 31.3% of the variability of the model, and also affected the autumn productivity in negative manner (β=-0.55).

Table 5. Best-fit (lowest AICc) linear regressions between autumn productivity and predictor variables. The predictor variables are: logarithm of enclosure size (lnSize), logarithm of pellet abundance index (lnPAI) and aerial predation index (API). All the significant models ($p<0.05$) are shown, and the significance of the variables included is indicated as follows: ** ($p<0.001$), * ($p<0.05$), no signal ($p>0.05$). Adjusted R^2 corresponds to the whole models.

Model	N	d.f.	Variables	AICc	ΔAICc	AICc weights	p	Adjusted R^2
Autumn productivity	11	2	lnSize**. lnPAI**	40.86	0	0.87	0.000	0.85
Autumn productivity	11	3	lnSize**. API. lnPAI**	44.71	3.86	0.13	0.000	0.85
Autumn productivity	11	1	lnPAI**	51.28	10.42	0.00	0.002	0.53
Autumn productivity	11	2	API. lnPAI*	53.55	12.69	0.00	0.006	0.53

The second model (LR3) only included lnPAI in the most parsimonious model (Table 6), so partial R^2 coincides with R^2 of the model, and the variable affected the spring productivity in negative manner (β=-0.72). As the set variable was not included in the best-fit model, we assumed no significant differences between the two sub-sets.

Table 6. Best-fit (lowest AICc) linear regressions between spring productivity and predictor variables. The predictor variables are: enclosure set, enclosure size, logarithm of pellet abundance index (lnPAI) and aerial predation index (API). All the significant models ($p<0.05$) are shown, and the significance of the variables included is indicated as follows: ** ($p<0.001$), * ($p<0.05$), no signal ($p>0.05$). Adjusted R^2 corresponds to the whole models.

Model	N	d.f.	Variables	AICc	ΔAICc	AICc weights	p	Adjusted R^2
Spring productivity	22	1	lnPAI**	121.24	0	0.37	0.000	0.50
Spring productivity	22	3	set. API. lnPAI**	123.21	1.96	0.14	0.000	0.51
Spring productivity	22	2	set. lnPAI**	123.27	2.03	0.13	0.000	0.48
Spring productivity	22	2	size. lnPAI**	123.41	2.16	0.12	0.000	0.48
Spring productivity	22	2	API. lnPAI**	123.56	2.32	0.12	0.000	0.47
Spring productivity	22	3	size. API. lnPAI**	125.18	3.93	0.05	0.000	0.47
Spring productivity	22	4	set. size. API. lnPAI**	125.92	4.68	0.04	0.000	0.49
Spring productivity	22	3	set. size. lnPAI**	125.93	4.69	0.04	0.001	0.45

Autumn productivity was excluded in the most parsimonious model (LR4, Table 7). As the only significant variable was lnPAI, partial R^2 coincides with R^2 of the model, and the variable affected the spring productivity in negative manner (β=-0.72).

Table 7. Best-fit (lowest AICc) linear regressions between spring productivity and two predictor variables: logarithm of pellet abundance index (lnPAI) and autumn productivity. All the significant models ($p<0.05$) are shown, and the significance of the variables included is indicated as follows: ** ($p<0.001$), * ($p<0.05$), no signal ($p>0.05$). Adjusted R^2 corresponds to the whole models.

Model	N	d.f.	Variables	AICc	ΔAICc	AICc weights	p	Adjusted R^2
Spring productivity	9	1	lnPAI**	47.92	0.00	0.74	0.010	0.50
Spring productivity	9	2	autumn productivity. lnPAI**	49.98	2.06	0.26	0.018	0.46

For practical purposes, we show the negative and logarithmic relationship between the initial rabbit density (estimated according to PAI and LR1), and both autumn and spring productivities (Figure 8). The steepest decrease in productivity occurred with between 10 and 40 rabbits per hectare.

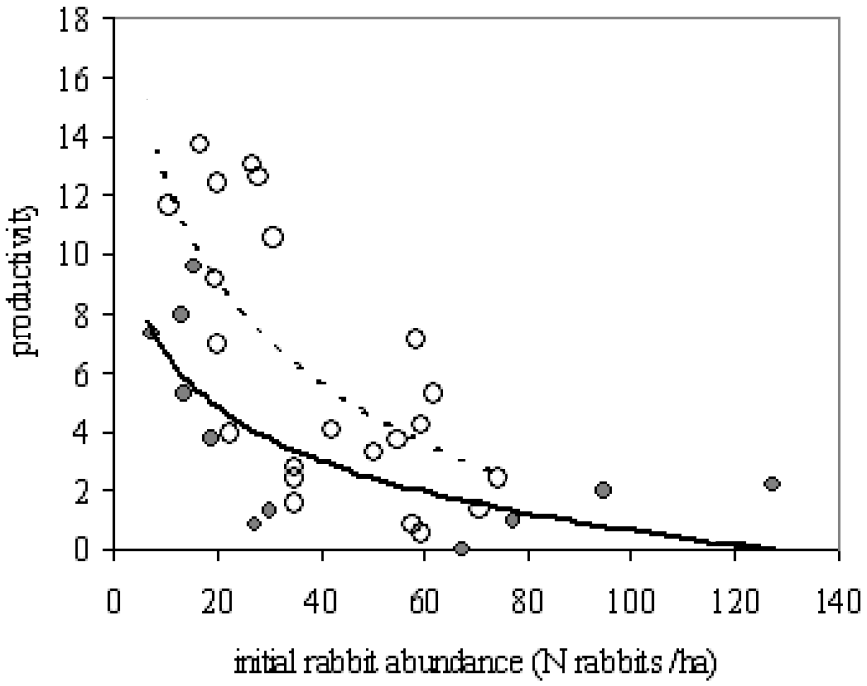

Figure 8. Linear regression for the initial rabbit density (rabbits/ha) and spring (white circles, N=22) and autumn (grey circles, N=11) productivity. Logarithmic fit-in for spring data (broken line). Logarithmic fit-in for autumn data (solid line).

Discussion

Rabbit abundance negatively affected the productivity within the restocking fenced plots, thus reducing their efficiency as extensive wild rabbit breeding systems. This result suggests that density-dependent factors were affecting the rabbit restocking enclosures at a population level, if we define density-dependence as negative feedback between rabbit abundance and population growth rate (Turchin, 1995, Turchin, 1999). This density-dependence may have been caused by two factors: a decrease in reproductive rates at higher densities, already reported in rabbits (Myers and Poole, 1962, Rödel et al., 2004b), but see (Trout and Smith, 1998), or an increase in mortality at higher densities, as has also been observed in rabbits (Rödel et al., 2004a).

The density-dependent adjustment of individual reproduction (Lack, 1954) and a lower proportion of individuals living in favourable places at high densities (Andrewartha and Birch, 1954) are the two major mechanisms that may explain a reduction in reproductive performance. Rödel *et al* (2004b) explained the reduced reproductive rates in higher densities of rabbits as a higher proportion of young females with a lower reproductive performance resulting from their poorer physical condition and lower social rank. Increased levels of aggressive interactions in a high density may also decrease fertility by diminishing gonadotropin secretion (Von Holst, 1998). On the other hand, density-dependent variation in juvenile mortality may be driven by extrinsic (diseases, trophic-level interactions) or intrinsic (physiology, behaviour) mechanisms (Rödel et al., 2004a). Social constraints may increase the energetic cost of subordinates, expose them to predators and cause chronic stress effects (Von Holst et al., 1999, Letty et al., 2000, Von Holst et al., 2002, DeVries et al., 2003, DeVries et al., 2007).

Enclosure size was also included in the autumn productivity model, although not in the spring model, with the highest productivity being in small enclosures,

contrary to the evidence which suggests that rabbits undergoing translocations to bigger enclosures will get better acclimated to their new environment (Rouco, 2008). Further research is advisable.

Another finding of this work was the lack of effect of autumn productivity on the following spring productivity. It has been suggested that in small mammals an increase in fecundity might occur only at the expense of future survivorship or future reproductive output (Speakman, 2008). However, our results did not show any effect of autumn productivity on further spring breeding, thus suggesting no limitation as regards productivity owing to the physiological cost of previous reproduction

In conclusion, we suggest that the management of rabbit restocking enclosures could be improved by releasing the surplus breeding stock from the enclosures before each breeding season in order to decrease density within the plot. Our data also suggest that if productivity is to be optimized, rabbit densities within the plots should remain below twenty to thirty rabbits per hectare.

CHAPTER 5

On the importance of mineralocorticoid receptors in social learning: modulation by social stimulus.

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Introduction

Aggressive behaviour has evolved as one of the most efficient means of competition in social species, which ensures individual survival and genetic success when resources are limited. As such, virtually all species have a neural system and a physical constitution that make them suitable for performing this (Haller and Kruk, 2006). When aggression is directed towards the monopolisation of limited resources through the defence of a territory, it becomes a major component of territorial behaviour (Maher and Lott, 1995, Maher and Lott, 2000). Various mechanisms have evolved in order to fulfil the defence function while reducing the cost derived from agonistic interactions. Versatile offensive behaviour (Haller et al., 1998a, Haller et al., 1998b), social organisation into hierarchies (Mock and Ploger, 1987) or winner and loser effects (Drummond and Canales, 1998) are associated with priming mechanisms (Schacter and Buckner, 1998), which makes the aggression response adaptable. It facilitates a differential response to the same stimulus - such as the intrusion of a conspecific into the territory - depending on the outcome of past conflicts.

Much of the aggression neurocircuitry overlaps with that of the hypothalamo-pituitary-adrenal (HPA) axis (Summers and Winberg, 2006), since aggression is both a stressor (Kruk et al., 2004) and one of the most immediate mechanisms by which stress enhances survival (Haller et al., 1998a, Mikics et al., 2007). The HPA axis represents a major evolutionary mechanism by which to maximize fitness through stress management (Selye, 1950, Blas et al., 2007): the perception of a stressor (Romero, 2004), regardless of its emotional valence (Buwalda et al., 2012), triggers a hormone cascade in the HPA axis which initiates and orchestrates the emergency life history stage (Wingfield et al., 1998), with the eventual release of glucocorticoid (GC) into the blood leading to changes in the structure and neurohistochemistry of the brain (de Kloet et al., 2005).

Their share in stress response signify that GC receptors play an important role in both the development and expression of aggressive behaviour (Haller et al., 1998b) and the storage of information (Oitzl et al., 1994). Some forms of GC receptors exert fast non-genomic effects from the plasmatic membrane of the cells, while cytosolic forms mediate slower and longer-term changes in synaptic plasticity and neural transmission by acting as transcription factors and altering gene activity and protein synthesis (Baker, 2003, Dallman, 2005). A short time-frame of action, independence of nuclear or cytosolic receptors and independence of genomic transcription and transduction mechanisms are the main three criteria by which to identify the non-genomic effects of GCs, which have immediate effects on brain function (Makara and Haller, 2001). The rapid aggressive behaviour of a rat towards an intruder fits into this operative definition of non-genomic effect (Makara and Haller, 2001, Mikics et al., 2004, Dallman, 2005), which is mediated by plasma membrane mineralocorticoid receptors (MR) (Joëls et al., 2008). Otherwise, genomic MRs maintain the integrity,

excitability and stability of the neuronal circuits, while genomic glucocorticoid receptors (GR) normalise the network activity and promote storage of the experience for future use (Joëls et al., 2008). The MR/GR balance hypothesis defends that homeostasis and health depend on the balanced interaction of these two genomic receptors. If the balance between onset and termination of the stress reaction is disturbed, then the individual loses its ability to maintain homeostasis if challenged (Avital et al., 2006, Joëls et al., 2008, Sousa et al., 2008).

In the context of social aggression, long-term physiological and behavioural effects of a single social stress experience have been reported in rats, both for the losers (Koolhaas et al., 1997) and the winners, and these have been referred to as priming experiences (Potegal, 1992). This priming is the basis of the precipitation and escalation of violent behaviour under stressful conditions, and also appears to be mediated by GC receptors (Kruk *et al*, *unpublished*). To date, the MR blockade has been observed to inhibit aggressive behaviour (Haller et al., 1998b), whilst in unpublished data, Kruk and colleagues have shown that it also prevents the long-lasting priming of hypothalamic attacks in naïve rats, which points to the important role of MRs in both the rapid non-genomic effect of GCs upon aggression, and the development of the neurocircuitry associated with the priming process. However, existing experiments concerning hypothalamic attack neglect both the causal mechanisms that trigger aggressive encounters and the effect of GC release upon the immediate perception of an emotional experience and the behavioural response (Ferris and Stolberg, 2010). We have therefore tested the effect of the MR blockade upon the escalation of aggressive behaviour in a more natural situation, using the resident-intruder paradigm.

The aim of this experiment is to test the effect of the MR blockade on the development of territorial offensive aggression, and the modulation of this development by the applied social stimulus.

Materials and Methods

Subjects and housing

Two batches of Wild type Groningen (WTG) rats (*Rattus norvegicus*) were used, each batch consisting of 21 rats. Their ancestors were originally wild-trapped animals, which were subsequently bred in our laboratory for 49 generations. These rats not only exhibit a rich repertoire of social behaviour, including aggression, but there is also a high degree of inter-individual variation. The WTG rats used in these experiments were 4 months old and had been housed in residential cages (80 x 55 x 40 cm) for a week with a sterilized female (from DEC 5873A) in order to facilitate residential offensive aggressive behaviour and prevent social isolation. The rats had free access to food and water throughout the experiments and were housed in climate-controlled rooms under a 12:12-hr inverted light–dark cycle. All experiments were performed during the dark phase (van der Vegt et al., 2003).

The role of the intruder during the aggressive confrontations was performed by male Wistar rats. Wistar rats were housed in large cages in social groups in order to avoid the combined effects of social defeat and social isolation. They were supplied with water and food *ad libitum* throughout the experiments. The first WTG batch was confronted with naïve Wistar rats, while the second WTG batch was confronted with previously defeated Wistar rats. The two batches of Wistar rats showed differences in their behaviour towards the resident WTG rats. The previously defeated rats were much more reactive after being placed in the resident’s cage. These differences in Wistar

behaviour resulted in differences in the aggressive behaviour between the two batches of resident WTG rats.

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC-RuG) of the University of Groningen.

Spironolactone injections

After a 1 week habituation period, half of the animals (10 from the first batch and 11 from the second batch) were subcutaneously injected with the MR blocker spironolactone (10 mg/kg) 1 hour prior to the first introduction of a male Wistar intruder. The other half of the animals was injected with vehicle solution (29% 2-hydroxypropyl-ß-cyclodextrin (RBI) in saline). The rats were injected once, prior the first encounter. They were injected at the beginning of the dark phase, since it has been found that blocking the MRs with spironolactone (5-10 mg/kg) during the early dark period dramatically and specifically reduces territorial aggression (Haller et al., 2000).

Aggression tests

All of the aggression tests were performed during the dark phase and under dim illumination, in the presence of an observer. For both batches, four aggression tests were performed on consecutive days, following the resident-intruder (RI) paradigm. For each test, the female was removed and an unfamiliar male conspecific (Wistar) was introduced into the home cage of the experimental rat (Haller et al., 1998b, van der Vegt et al., 2003). The attack latency time (ALT) was scored in all the encounters, with the test being terminated shortly after occurrence of an attack (days 2 and 3) or after a maximum test duration of 10 min (days 1 and 4). During the first and fourth tests, the full behavioural profile was video recorded starting at the introduction of the intruder and for 10 min after the first attack or for a maximum of 10 min if there was no attack.

Behavioural variables

The following behavioural elements were scored: attack latency time (ALT), social aggressive behaviour (clinch, threat, offensive upright, keep down, chase), social non-aggressive behaviour (investigate opponent, sniff in anogenital region, social groom, mount), exploratory behaviour (explore environment, rear), immobility, and groom (van der Vegt et al., 2003).

Statistical analysis

Recorded behavioural observations were explored using E-line software (custom made), and statistically analysed to check for treatment (spironolactone *vs* vehicle), batch (naïve *vs* defeated intruders) and escalation (days 1 to 4) effects.

In order to analyse ALT data in a conservative manner, we divided the dataset into two blocks, which were then analysed separately: the first block included the encounters in which no attack was observed during the maximum test duration (ALT>600 s, N=35), and the second included the encounters in which attacks were observed (ALT<600 s, N=121). We analysed the distribution of non-attack encounters (ALT>600 s) as regards the different treatments, batches and days by using Chi-square analysis. We then used linear mixed models to check for differences between treatments, batches and days in the ALT<600 s dataset. The repeated samplings per individual were accommodated with the inclusion of the individual (N=42) as a random factor. The fixed components used to explain the response variable were the treatment (N=2), batch (N=2), day (N=4) and their interactions. The ALT of each resident-intruder encounter was the response variable. In order to normalise the response variable, we transformed ALT data with root square. The addition of a variance structure which allowed for heterogeneity of variance between treatments and batches improved the final model. The residuals of the final model did not show temporal correlations, and the Q-Q plot suggested a normal distribution of the residuals,

signifying that both normality and independence were confirmed (Zuur et al., 2009). Other behavioural variables were analysed using generalized lineal models to check for differences between treatments, batches and days (day 1 *vs* day 4). Table 8 shows the error distribution and data transformations used in each model. When required by the models, variance structures were added to account for the heterogeneity in the residual spread between treatments, batches and days.

Table 8. Error distribution, data transformation and the levels throughout which heterogeneity of residuals was allowed are shown for every behavioural variable analysed by generalized linear models.

Behavioural variables		Distribution	Transformation	Heterogeneity of residuals
N. attacks		Poisson	log	-
Social aggressive	total	Normal	-	Batch
behaviour (%)	threat	Normal	-	Treatment, Day
	keep down	Normal	log	Batch
	clinch	Normal	log	Treatment, Day
	chase	Normal	log	Treatment, Day, Batch
	offensice upright	Normal	log	Treatment, Day, Batch
Social non-aggressive behaviour (%)		Normal	log	Treatment
Exploratory	cage exploration	Normal	-	Day
behaviour (%)	rearing	Normal	log	Treatment
Groomming (%)		Normal	log	Treatment
Immovility (%)		Binomial	logistic	-

The model selection was based on Akaike's Information Criterion (AIC). The model diagnosis was based on analyses of model residuals. The statistical analyses were performed using R 2.9 software (Team, 2008), and the *nlme* package (Pinheiro and Bates, 2000) was used to fit the models.

Results

Spironolactone injections reduced the variability in ALT in the first batch, increasing it on the first day and decreasing it on successive days (Figure 9, a). However, the effect was not as great in the second batch (Figure 9, b).

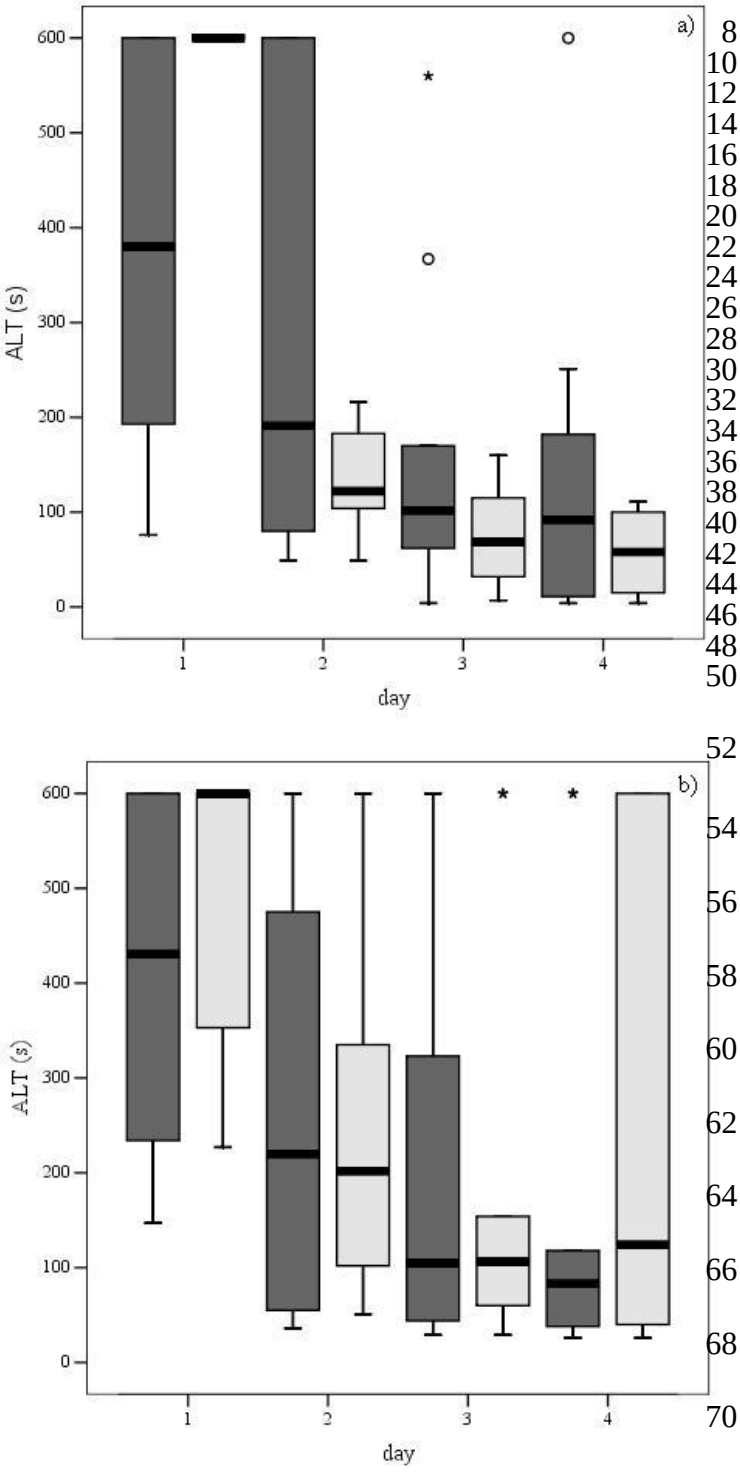

Figure 9. ALT scores by batches 1 (a) and 2 (b) on days 1 to 4. Scores from the groups treated with spironolactone are shown with empty boxes and, those from control groups, with grey boxes. Medians are represented as black horizontal bars, first and third quartiles as box boundaries, maximum and minimum observed values as vertical lines, outliers as circles and extreme values as stars. The upper boxplot represents the first batch's results (a), and the lower boxplot, the second batch's results (b). All the ALT data are included, both non attackers (ALT=600 s on this figure) and attackers (ALT<600 s) scores.

Non attackers were not randomly distributed across treatments, batches, and days (Chi-square, χ^2=42.5, df=16, p=0.0003). Spironolactone injections completely abolished the attacks in the first batch on the first day, while all the individuals attacked in less than 600 s on successive days (Table 9).

Table 9. The proportion of WTG rats which did not attack the Wistar intruders during the 600 s duration of the resident-intruder experiment ($N_{ALT>600}/N_{total}$). The proportions are displayed according to the different categories tested: the treatment (vehicle *vs* spironolactone), the Wistar intruders (batch 1 *vs* batch 2) and the consecutive days (days 1 to 4).

	Vehicle		Spironolactone	
Day	batch 1	batch 2	batch 1	batch 2
1	0.4	0.3	1	0.6
2	0.3	0.2	0	0.2
3	0	0.1	0	0.2
4	0.1	0	0	0.2

The linear mixed models applied to the second ALT dataset (ALT<600 s) showed significant differences between days (ALT_1=293 ± 132 SD, ALT_2=167 ± 113 SD, ALT_3=127 ± 134 SD, ALT_4=92 ± 67 SD; ANOVA, F=103.04, df=1,78, p<0.0001) and between treatments ($ALT_{vehicle}$=175 ± 145 SD, $ALT_{spironolactone}$=122 ± 98 SD; ANOVA, F=3.70, df=1,35, p=0.06).

The spironolactone also affected a different array of behaviours. On the first day, the attack behaviour (number of attacks and % clinch, Table 10) was completely abolished by the spironolactone in the first batch, and overall social aggressive behaviour, threatening, clinching and chasing behaviours were significantly reduced, while social exploratory behaviour was significantly incremented. On the fourth day, however, the effect of the spironolactone injections was distinctly different in the first

batch, with an increase in the number of attacks and overall aggressive behaviour. The second batch's treatment effects were not as strong as the first batch's.

Table 10. Behavioural variable results are shown for the different treatments, the two batches and days 1 and 4. The scores represent the percentage of time spent on each specific behaviour. Significant differences ($p<0.05$) between levels are indicated as * (vehicle *vs* spironolactone), # (batch 1 *vs* batch 2) and + (day 1 *vs* day 4) throughout the variables.

		Day 1				Day 4			
		vehicle		spironolactone		vehicle		spironolactone	
Behavioural variables		Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
N. attacks #+		6.8	2.4	0	1.7	16.7	8.2	24	8.2
Social aggressive	total	33.3	17.5	17.6	16.4	54.4	44.9	66.8	39.8
behaviour (%)	threat *+	19.5	14.1	1.8	12	42.3	37.2	49.1	34.1
	keep down #	6.8	0.8	10.5	1.9	3.9	1.7	7.1	2.3
	clinch *+	0.9	0.5	0	0.2	2.3	1.4	4.7	0.9
	chase *#	1.7	0.2	0.1	0.4	3.2	3.2	4.6	2.5
	offensice upright	4.6	2	5	1.8	3.5	1.5	2.2	1.9
Social non-aggressive behaviour (%)*+		30.7	20.4	58	35.1	6.3	15	9.6	12.6
Exploratory	cage exploration #	16.7	26.8	11	27.1	23.6	28.4	17.7	23.7
behaviour (%)	rearing *	5.2	6.2	0.2	7	3.5	4.3	2.5	3.1
Groomming (%) #		10.7	27.3	5.2	10.6	10.9	6.6	3	15.5
Immovility (%)		3.3	1.8	7.7	3.8	1.4	1	0.7	3.9

Discussion

Our results suggested that the MR blockade in naïve WTG rats may affect the escalation of aggressive behaviour under stressful conditions, in the context of a territorial conflict. The results also suggested that this effect may depend on the motivation to initiate offensive aggressive behaviour in the context of the resident-intruder paradigm.

When naïve intruders were used, the MR blockade by spironolactone completely abolished the immediate attack behaviour, while it produced a distinctly different effect in the long-term, since the attack behaviour increased significantly and the attack latency time was reduced. The limited ALT variability in both situations (abolishment of attack behaviour in the short term and the enhancement of this behaviour in the long term) points to a reduction in neuroplasticity (Kim and Diamond, 2002) associated with the spironolactone injections. This reduction in neuroplasticity may have been related to the long term depression (LTD) described in hippocampal neurons when exposure to acute stressors was combined with the MR blockade by spironolactone (Avital et al., 2006). When previously defeated intruders were used, MR blockade by spironolactone failed to completely abolish the immediate attack behaviour, and the variability in ALT and other behavioural traits was also higher than in that of the first batch. However, after non attackers were excluded from the analysis, no significant differences were observed between batches. The apparent disposition of the second batch individuals to attack the intruders more easily during their first encounter could be explained by the scared and reactive behaviour of the previously defeated Wistar rats, which, with their behaviour, triggered the attacks of spironolactone-treated WTG rats.

It was noteworthy that, while the aggressive behaviour measured in the successive encounters is driven by fast non-genomic mechanisms (Makara and Haller, 2001, Mikics et al., 2004, Dallman, 2005), the priming process, the consequences of which are observed in later exposures, is associated with genomic mechanisms, which are most probably mediated by genomic MRs. Spironolactone lasts less than 12 hours in rats (Sadée et al., 1974), so MRs were blocked only during the first day encounter, which explains the inhibition of attack behaviour (Haller et al., 1998b). These receptors

were not blocked during the encounters on days 2-to-4, signifying that the differences between spironolactone and control groups were probably related to long-lasting (genomic) changes that took place during those 12 hours after the first single injection. The genomic effects of the MR blockade were thus reflected in non-genomic processes measured in the encounters which took place on days 2-to-4. Furthermore, the behavioural effects were different when the rats were confronted with naïve or previously defeated intruders, suggesting that the genomic effect reflected in the priming of aggressive behaviour was affected by the motivation to be aggressive during the first confrontation with an intruder.

On the other hand, while the abolition of attack behaviour by the MR blockade is supported by previous studies (Haller et al., 1998b), the inverse long-term effect observed in this work is not as intuitive as that. The stress mechanism aims to produce an optimisation of the aggressive response in order to reduce the cost derived from agonistic interactions, as was explained in the introduction. Why then, should the learning process enhance the attack behaviour, well above the escalation observed in the control group? The MR blockade during a stressful event may have impaired the stress responsiveness and the behavioural programming by MR/GR imbalance (Avital et al., 2006, Sousa et al., 2008), because GCs are necessary for the memory formation that follows an acute stressful experience (Sapolsky et al., 2000, Beylin and Shors, 2003), and the blockade of MRs could therefore have diminished the social learning process. Furthermore, the hippocampus, a structure involved in memory processing and HPA axis regulation, has one of the highest concentrations of GC receptors in the mammalian brain, which, during our experiment, did not act in balance with MR occupation by spironolactone.

Neural systems involved in learning, memory and decision making are most sensitive to acute stress arousals, since they enhance the chances of survival, and these cognitive events are reinforced by genomic mechanisms affecting long-term behaviour, probably via the induction of morphological and neurochemical changes (Ferris and Stolberg, 2010). In this study we observed that the blockade of the stress response during the first aggressive interaction had long-term effects upon the behavioural response to future conflicts. Changes in the effect of an immediate rise in corticosterone release during the first conflict upon long-term neurochemical and morphological modifications may alter the perception of stressors and the behavioural response to them by means of changes in synaptic networks (Avital et al., 2006) or receptor density (Sapolsky et al., 1986). We also observed that the effect of the MR blockade varied depending on the motivation to show aggressive behaviour immediately after spironolactone administration, and that the variability in the behavioural response was enhanced by the use of previously defeated intruders, which points to the role of the perception of a stressor on the escalation of aggressive behaviour.

GENERAL DISCUSSION

As summarised in the Introduction section of this thesis, wild rabbit restocking is a management tool that is widely used to boost rabbit populations in the areas in which the species has a conservational or hunting value, but remains scarce. The economic and ecological importance of the European rabbit in the Iberian Peninsula signifies that the scientific research focused on this species and its management is abundant in literature. However, scientific papers which regard intrinsic aspects of wild rabbits, such as their social organisation, chemical communication or physiological stress, are secondary to those which regard extrinsic factors such as predation, diseases or habitat characteristics. This relegation of rabbit sociobiology to a secondary level may be in part explained by the methodological constraints of its study. The analysis of chemical components is laborious, expensive, and requires specialised training, while the observation of individual behaviour is laborious and difficult to achieve in the wild.

Our results suggest that Near Infrared Reflectance Spectroscopy and indigestible faecal markers are techniques that could feasibly be applied to the study of territorial and scent marking behaviour as regards the ease with which they can be applied to field studies and their low cost, which could help to make the study of wild rabbit sociobiology available to the average researcher. It is encouraging to note that physiological stress has been studied in wild rabbits for the last few years, although these studies still remain scarce, and I have therefore attempted to make a contribution to this field in the form of a preliminary study concerning the acute stress response in wild rabbits and its predictive value in restocking programs (see Chapter 3).

When applied to faecal analysis, Near Infrared Reflectance Spectroscopy - F.NIRS - was able to detect territorial marking signals in wild rabbit faeces, provided

that the variability associated with phenology was reduced. There are two implications of this study: first, that it would be difficult to develop a single model for the identification of territorial marking signals in rabbit faeces owing to the variability in the composition of the faecal matrix (associated with variability in diet throughout the year) and in the intensity of the scent-marking signal; and second, upon acknowledging the fact that a F.NIRS model would predict the characteristics of samples collected from the same sample pool (environment, population, season) from which the model samples were collected (the bigger the model, the wider the predictability of samples), the method is valid, cheap, easy, non-invasive and user-friendly, and open up the possibilities of the non-invasive monitoring of chemical communication by wild rabbit societies. With regard to the use of faecal colorants on wild rabbits, the rabbits' double-digestion system has probably constrained the availability of markers (see Chapter 2). However, our results were encouraging since we were able to colour-mark different social territories in a rapid, cheap and easy manner, without disturbing the animals and with no need to monitor the individuals. Furthermore, our data showed a variability in latrine distribution which could not have been detected by the traditional latrine mapping.

These methodological studies were also relevant in a different way, since they revealed an active social behaviour in enclosed wild rabbit populations, which, despite its being thoroughly described in prior studies (Mykytowycz, 1958, Mykytowycz, 1959b, Mykytowycz, 1959a, Lockley, 1961, Mykytowycz and Gambale, 1965, Mykytowycz and Gambale, 1969), is usually neglected in rabbit management programs. The maximum marking activity areas showed that the rabbits defended warrens, food supply points and territorial boundaries (see Chapter 2), and this observation could easily be associated with the density dependence described in Chapter 4: social

behaviour may be a constraint to the efficiency of wild rabbit restocking plots, and should be regarded as such in management programs. The gregariousness and territoriality of wild rabbits will also most probably condition the manner in which such populations will expand and colonize new areas and, as the colonization of new areas by the restocked rabbits is the expected final outcome of the whole restocking process (except when restocking plots are built as supplementary feeding stations for predators), more in-depth research into their sociobiology is mandatory if an efficient management is to be achieved. The aim of the restocking process should therefore condition the management measures applied to the translocated rabbits' plots, since if their aim is the establishment and maintenance of highly productive populations, social stability and moderate rabbit densities should be pursued (see Chapter 4), while if the establishment of predator feeding plots is the major aim of the restocking actions, higher abundance may be useful.

Moreover, the neurocircuitry for social aggression and stress are closely interrelated (see Chapter 5), since aggression is both a stressor and one of the most immediate means by which stress enhances survival. Hence, as Chapter 5 suggests, the stress mechanism is necessary to develop social hierarchies, while social hierarchies are simultaneously a source of stress for the individuals that are a part of it (Von Holst et al., 1999, Letty et al., 2000, Von Holst et al., 2002, DeVries et al., 2003, DeVries et al., 2007). In Chapter 5, I tested a mineralocorticoid antagonist (spironolactone) as a potential drug to inhibit social aggression in rats (potentially and hopefully exportable to wild rabbits). This drug acted by blocking the HPA axis response, which elicited aggression in the context of the defence of a territory. Various drugs have been tested in wildlife restocking, conservation and research programs (Ramdohr et al., 2001, Golden et al., 2002, Mentaberre et al., 2010). In wild rabbits, an attempt has been made to use

β-blockers to reduce the short-term mortality rates associated with translocation stress, but no effect on survival has been detected (Letty et al., 2000). In our experiment, the spironolactone acted by completely abolishing social aggression in the short term, but enhancing it in the long term, which probably suggests the important role of the HPA axis response in the modulation of social aggression. This result could be explained in part by the fact that glucocorticoids, far from limiting activity in the enhancement of the stress response, contribute to a recovery from it and facilitate the storage of information for future conflicts (Sapolsky et al., 2000). The use of drugs that interfere with a correct functioning of the HPA axis response may not therefore be advisable in the long term.

This correct functioning of the HPA axis involves low baseline glucocorticoid levels, a fast increase of glucocorticoids, and a rapid induction of negative feedback. A rapidly initiated and rapidly cleared stress response is presumably healthy for the individual. However, the coexistence of high and low responders in the same environment suggests that this may not be so for the populations' fitness in the long term (Blas et al., 2007, Breuner et al., 2008). In a preliminary study (Chapter 3), I have attempted to shed light upon the relationship between responsiveness to stressors and the growth of enclosed wild rabbit populations. The relationship was U-shaped, as expected in a homeostatic mechanism such as the stress response, with maximum productivity values corresponding to reactivity levels of between 100 and 150%. The highly responsive samples showed a marked decrease in annual productivity, suggesting that either a fast and high stress response does not favour population growth, or that the environmental conditions which induce reactivity levels to above 150% could be related to a lower population growth. Whatever the case, the non invasive measurement of reactivity to stressors may be a feasible method by which to predict the growth of wild rabbit populations during the breeding season.

CONCLUSIONS

1. The Faecal Near Infrared Reflectance Spectroscopy -F.NIRS- is a suitable method with which to detect territorial marking signals in wild rabbit pellets. This new, easy, cheap, fast and non-invasive method may therefore contribute to studies concerning scent-marking behaviour by wild rabbits.

2. Phenology seems to be one of the principal sources of error in the detection of marking signals in pellets by F.NIRS, which probably affects both the chemical composition of the pellets and the intensity of the marking signals. Another source of error is the age of the pellet, since the signal detected by F.NIRS lasts for less than one week, suggesting a low half life for the markings substances in pellets.

3. The indigestible faecal colour markers are a useful tool with which to explore the territorial behaviour of wild rabbits, in addition to providing easy clues with which to define spatial frameworks based on social structures.

4. We used indigestible faecal markers to identify different maximum latrine-marking activity zones in different enclosures, suggesting different solutions to the trade-off between the cost of defending a territory and the benefits obtained from resource monopolisation. The nuclei of the marking areas corresponded, respectively, to the warren, the between-warren area and the food supply point.

5. The analysis of faecal corticosterone metabolites enabled the measurement of a continuous range of reactivity to acute stressors and the detection of different responses by different populations in a non-invasive manner.

6. The variability in the stress response was related to the variability in the wild rabbit populations' annual productivity. Hence, wild rabbit populations' growth could be predicted by means of non-invasive measures of the acute stress response.

7. Rabbit abundance negatively affected the productivity within the restocking fenced plots, thus reducing their efficiency as extensive wild rabbit breeding systems. The management of rabbit restocking enclosures could therefore be improved by releasing the surplus breeding stock from the enclosures before each breeding season in order to maintain rabbit densities below twenty to thirty rabbits per hectare.

8. The blockade of mineralocorticoid receptors in naïve wild-type rats affected the escalation of aggressive behaviour under stressful conditions, in the context of a territorial conflict. This effect depended on the motivation to initiate offensive aggressive behaviour in the context of the resident-intruder paradigm.

9. The blockade of mineralocorticoid receptors enhanced the territorial aggressions in the long term. It is not therefore advisable to apply this method in wild rabbits as a means to facilitate the introduction of restocked rabbits into the territories of resident groups.

REFERENCES

Akaike, H., 1974. A New Look at the Statistical Model Identification. IEEE Transactions on Automatic Control. AC-19, 716-723.

Alberts, A., 1992. Constraints on the design of chemical communication system in terretrial vertebrates. Am. Nat. 139, 62-89.

Andrewartha, H. G. and Birch, L. C., 1954. The distribution and abundance of animals. Univ. of Chicago Press.,

Angulo, E., 2003. Factores que afectan a la distribución y abundancia del conejo en Andalucía. Universidad Complutense de Madrid,

Arenas, A. J., Astorga, R. J., García, I., Varo, A., Huerta, B., Carbonero, A., Cadenas, R. and Perea, A., 2006. Captive Breeding of Wild Rabbits: Techniques and Population Dynamics. Journal of Wildlife Management 70, 1801-1804.

Argüello, J. L., Llanos, A. and Pérez-Ordoyo, L. I., 1988. Enfermedad vírica hemorrágica del conejo en España. Medicina Veterinaria. 5, 645-650.

Avital, A., Segal, M. and Richter-Levin, G., 2006. Contrasting roles of corticosteroid receptors in hippocampal plasticity. The Journal of Neuroscience. 26, 9130-9134.

Baker, M. E., 2003. Evolution of adrenal and sex steroid action in vertebrates: a ligand-based mechanism for complexity. BioEssays 25, 396-400.

Barnes, R. J., Dhanoa, M. S. and Lister, S. J., 1989. Standard Normal Variate Transformation and De-trending of Near-Infrared Diffuse Reflectance Spectra. Appl. Spectrosc. 43, 772-777.

Barrio, I. C., 2010. PhD Thesis: The rabbit as an agricultural pest in its native distribution range. Universidad de Castilla La Mancha,

Barrio, I. C., Acevedo, P. and Tortosa, F. S., 2010a. Assessment of methods for estimating wild rabbit population abundance in agricultural landscapes. Eur J Wildl Res. 56, 335-340.

Barrio, I. C., Bueno, C. G. and Tortosa, F. S., 2009. Improving predictions of the location and use of warrens in sensitive rabbit populations. Animal Conservation. 12, 426-433.

Barrio, I. C., Bueno, C. G. and Tortosa, F. S., 2010b. Alternative food and rabbit damage in vineyards of southern Spain. Agriculture, Ecosystems and Environment. 138, 51-54.

Beylin, A. V. and Shors, T. J., 2003. Glucocorticoids are necessary for enhancing the acquisition of associative memories after acute stressful experience. Hormones and Behavior. 43, 124-131.

Blas, J., Bortolotti, G. R., Tella, J. L., Baos, R. and Marchant, T. A., 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. Proceedings of the National Academy of Sciences of the United States of America 104, 8880-8884

Bonier, F., Martin, P. R., Moore, I. T. and Wingfield, J. C., 2009. Do baseline glucocorticoids predict fitness? Trends in Ecology and Evolution. 24, 634–642.

Boval, M., Coates, D. B., Lecomte, P., Decruyenaere, V. and Archiméde, H., 2004. Faecal near infrared reflectance spectroscopy (NIRS) to assess chemical composition, in vivo digestibility and intake of tropical grass by Creole cattle. Anim. Feed Sci. Tech. 114, 19-29.

Branco, M., Ferrand, N. and Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity. 85, 307-317.

Breuner, C. W., Patterson, S. H. and Hahn, T. P., 2008. In search of relationships between the acute adrenocortical response and fitness. General and Comparative Endocrinology 157, 288-295.

Burnham, K. P., Anderson, D. R. and Huyvaert, K. P., 2011. AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. Behavioral Ecology and Sociobiology 65, 23-35.

Burns, D. A. and Ciurczak, E. W., 2001. Handbook of Near-Infrared Analysis. Second Edition, Revised and Expanded. Marcel Dekker, Inc.,

Busch, D. S. and Hayward, L. S., 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation. 142, 2844–2853

Buwalda, B., Scholte, J., de Boer, S. F., Coppens, C. M. and Koolhaas, J. M., 2012. The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. Hormones and Behavior. 61, 218-226.

Cabezas, S., Blas, J., Marchant, T. A. and Moreno, S., 2007. Physiological stress levels predict survival probabilities in wild rabbits. Hormones and Behavior. 51, 313-320.

Cabezas, S., Calvete, C. and Moreno, S., 2011. Survival of translocated wild rabbits: importance of habitat, physiological and immune condition. Animal Conservation. 14, 665-675.

Cabezas, S. and Moreno, S., 2007. An experimental study of translocation success and habitat improvement in wild rabbits. Animal Conservation. 10, 340-348.

Calvete, C., 2006. Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease. Conservation Biology. 20, 1232-1241.

Calvete, C., Angulo, E., Estrada, R., Moreno, S. and Villafuerte, R., 2005. Quarantine length and survival of translocated European wild rabbits. Journal of Wildlife Management. 69, 1063-1072.

Calvete, C. and Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving the release protocol. Biological Conservation. 120, 507-516.

Calvete, C., Estrada, R., Angulo, E. and Cabezas-Ruiz, S., 2004a. Habitat factors related to wild rabbit conservation in an agricultural landscape. Landscape Ecology. 19, 531-542.

Calvete, C., Estrada, R., Lucientes, J., Osácar, J. J. and Villafuerte, R., 2004b. Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits. Veterinary Record. 155, 388-392.

Calvete, C., Estrada, R., Villafuerte, R., Osácar, J. J. and Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Veterinary Record. 150, 776-782.

Calvete, C., Pelayo, E. and Sampietro, J., 2006. Habitat factors related to wild rabbit population trends after the initial impact of rabbit haemorrhagic disease. Wild. Res. 33, 467-474.

Calvete, C., Villafuerte, R., Lucientes, J. and Osácar, J. J., 1997. Effectiveness of traditional wild rabbit restocking in Spain. Journal of Zoology. 241, 271-277.

Carter, A., Luck, G. W. and McDonald, S. P., 2012. Ecology of the red fox (Vulpes vulpes) in an agricultural landscape. 2. Home range and movements Australian Mammalogy. 34, 175-187.

Catalán, I., Rodríguez-Hidalgo, P. and Tortosa, F. S., 2008. Is habitat management an effective tool for wild rabbit (*Oryctolagus cuniculus*) population reinforcement? European Journal of Wildlife Research. 54, 449-453.

Ciurczak, E. W., 2001. Principles of Near-Infrared Spectroscopy. In: Burns DA and Ciurczak EW (ed) Handbook of Near-Infrared Analysis. Marcel Dekker, Inc., New York.

Cowan, D. P., 1987. Group living in the European rabbit (*Oryctolagus cuniculus*): mutual benefit or resource localization? Journal of Animal Ecology. 56, 779-795.

Chrousos, G. P., 2009. Stress and disorders of the stress system. Nature Reviews Endocrinology. 5, 374–381.

Dallman, M. F., 2005. Fast glucocorticoid actions on brain: Back to the future Frontiers in Neuroendocrinology. 26, 103-108.

Dallman, M. F., Akana, S. F., Bradbury, M. J., Strack, A. M., Hanson, E. S. and Scribner, K. A., 1994. Regulation of the hypothalamo-pituitary-adrenal axis during stress: feedback, facilitation and feeding. Seminars in Neuroscience 6, 205–213.

de Kloet, E. R., Joëls, M. and Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nature Reviews-Neuroscience. 6, 463-475.

Delahay, R. J., Brown, J. A., Mallinson, P. J., Spyvee, P. D., Handoll, D., Rogers, L. M. and Cheeseman, C. L., 2000. The use of marked bait in studies of the territorial organization of the European Badger (Meles meles). Mammal Review. 30, 73-87.

Delibes-Mateos, M., Farfán, M. A., Olivero, J., Márquez, A. L. and Vargas, J. M., 2009. Long-term changes in game species over a long period of transformation in the Iberian Mediterranean landscape. Environmental Management.

Delibes-Mateos, M., Farfán, M. A., Olivero, J. and Vargas, J. M., 2010. Land-use changes as a critical factor for long-term wild rabbit conservation in the Iberian Peninsula. Environmental Conservation 37, 1-8.

Delibes-Mateos, M., Ferreras, P. and Villafuerte, R., 2008b. Rabbit populations and game management: the situation after 15 years of rabbit haemorrhagic disease in central-southern Spain. Biodiversity and Conservation. 17, 559-574.

Delibes-Mateos, M., Redpath, S. M., Angulo, E., Ferreras, P. and Villafuerte, R., 2007. Rabbits as a keystone species in southern Europe. Biological Conservation. 137, 149-156.

Delibes-Mateos, M., Smith, A. T., Slobodchikoff, C. N. and Swenson, J. E., 2011. The paradox of keystone species persecuted as pests: A call for the conservation of abundant small mammals in their native range. Biological Conservation. 144, 1335-1346.

Delibes, M. and Hiraldo, F., 1981. The rabbit as prey in the iberian mediterranean ecosystem. I World Lagomorph Conference, University of Guelph, Ontario.

DeVries, A. C., Craft, T. K. S., Glasper, E. R., Neigh, G. N. and Alexander, J. K., 2007. Review: 2006 Curt P. Richter award winner. Social influences on stress responses and health. Psychoneuroendocrinology 32, 587–603.

DeVries, A. C., Glasper, E. R. and Detillion, C. E., 2003. Social modulation of stress responses. Physiology & Behavior. 79, 399-407.

Dickens, M. J., Delehanty, D. J. and Romero, L. M., 2010. Stress: An inevitable component of animal translocation. Biological Conservation. 143, 1329-1341.

Dixon, R. and Coates, D., 2009. Review: Near infrared spectroscopy of faeces to evaluate the nutrition and physiology of herbivores. J. Near Infrared Spectrosc. 17, 1-31.

Doty, R. L., 1986. Odor-guided behaviour in mammals. Experientia. 42, 257-271.

Drummond, H. and Canales, C., 1998. Dominance between booby nestlings involves winner and loser effects. Animal Behaviour. 55, 1669-1676.

Eldridge, D. J., Constantinides, C. and Vine, A., 2006. Short-term Vegetation and Soil Responses to Mechanical Destruction of Rabbit (*Oryctolagus cuniculus* L.) Warrens in an Australian Box Woodland. Restoration Ecology. 14, 50-59.

Eldridge, D. J. and Simpson, R., 2002. Rabbit (*Oryctolagus cuniculus* L.) impacts on vegetation and soils, and implications for management of wooded rangelands. Basic and Applied Ecology. 3, 19-29.

Evans, D. G., Scotter, C. N. G., Day, L. Z. and Hall, M. N., 1993. Determination of the authenticity of orange juice by discriminant analysis of near infrared spectra. A study of pretreatment and transformation of spectral data. J. Near Infrared Spectrosc. 1, 33-44.

Farfán, M. A., Vargas, J. M., Guerrero, J. C., Barbosa, A. M., Duarte, J. and Real, R., 2008. Distribution modelling of wild rabbit hunting yields in its original area (S Iberian Peninsula). Italian Journal of Zoology. 75, 161-172.

Fernández-de-Simón, J., Díaz-Ruiz, F., Cirilli, F., Tortosa, F. S., Villafuerte, R., Delibes-Mateos, D. and Ferreras, P., 2011. Towads a standardized index of European rabbit abundance in Iberian Mediterranean habitats. Eur J Wildl Res. 57, 1091-1100.

Fernández-Olalla, M., Martínez-Jauregui, M., Guil, F. and Miguel-Ayanz, A. S., 2010. Provision of artificial warrens as a means to enhance native wild rabbit populations: What type of warren and where should they be sited? European Journal of Wildlife Research. 56, 829-837.

Ferrand, N., 2008. Inferring the evolutionary history of the European rabbit (Oryctolagus cuniculus) from molecular markers. In: Alves, P. C., Ferrand, N. and Hackländer, K., Lagomorph Biology: Evolution, Ecology and Conservation. Springer-Verlag, pp. 47-63.

Ferreira, C. and Delibes-Mateos, D., 2010. Wild Rabbit Management in the Iberian Peninsula: state of the art and future perspectives for iberian lynx conservation. Wild. Biol. in practice. 3, 48-66.

Ferris, C. F. and Stolberg, T., 2010. Imaging the immediate non-genomic effects of stress hormone on brain activity. Psychoneuroendocrinology 35, 5-14.

Fuller, G., Margulis, S. W. and Santymire, R., 2010. The Effectiveness of Indigestible Markers for Identifying Individual Animal Feces and Their Prevalence of Use in North American Zoos. Zoo Biology 29, 1-20.

Galaverni, M., Palumbo, D., Fabbri, E., Caniglia, R., Greco, C. and Randi, E., 2012. Monitoring wolves (Canis lupus) by non-invasive genetics and camera trapping: A small-scale pilot study. European Journal of Wildlife Research. 58, 47-58.

Gálvez, L., 2008. El conejo (*Oryctolagus cuniculus*) como ingeniero de ecosistemas. Universidad de Alcalá,

Gidenne, T., 1992. Effect of fibre level, particle size and adaptation period on digestibility and rate of passage as measured at the ileum and in the faeces in the adult rabbit. Br. J. Nutr. 67, 133-146.

Gil-Sanchez, J. M., 2011. Resultados de las Actuaciones de mejora de conejo dirigidas a la conservación del lince ibérico en Andalucía. II Internacional European rabbits congress, Toledo.

Gobert, G. N., Chai, M., Duke, M. and McManus, D. P., 2005. Copro-PCR based detection of Schistosoma eggs using mitochondrial DNA markers. Mol Cell Probes. 19, 250-254.

Golden, H. N., Shults, B. S. and Kunkel, K. E., 2002. Immobilization of Wolverines with Telazol® from a Helicopter. Wildlife Society Bulletin. 30, 492-497.

González-Redondo, P., 2003. Contribución al conocimiento de la producción del conejo silvestre (Oryctolagus cuniculus) en cautividad estricta. University of Córdoba,

González-Redondo, P., 2009. Injuries in cage-bred wild rabbits (Oryctolagus cuniculus) caused by the size of the orifices in the cage floor. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 61, 1246-1250.

González-Redondo, P. and Zamora-Lozano, M., 2008. Neonatal cannibalism in cage-bred wild rabbits (Oryctolagus cuniculus). Archivos de medicina veterinaria. 40, 281-287.

Goodrich, B. S., Hesterman, E. R., Murray, K. E., Mykytowycz, R., Stanley, G. and Sgowdz, G., 1978. Identification of behaviorally significant volatile compounds in the anal gland of the rabbit, Oryctolagus cuniculus. J. Chem. Ecol. 4, 581-594.

Goodrich, B. S., Hesterman, E. R., Shaw, K. S. and Mykytowycz, R., 1981. Identification of some volatile compounds in the odor of fecal pellets of the rabbit, *Oryctolagus cuniculus*. J. Chem. Ecol. 7, 817-827.

Goodrich, B. S. and Mykytowycz, R., 1972. Individual and sex differences in the chemical composition of pheromone-like substances from the skin glands of the rabbit, Oryctolagus cuniculus. J. Mammal. 53, 540-548.

Gump, B. B. and Matthews, K. A., 1999. Do Background Stressors Influence Reactivity to and Recovery From Acute Stressors? Journal of Applied Social Psychology. 29, 469–494.

Haller, J., Halasz, J., Makara, G. B. and Kruk, M. R., 1998a. Acute effects of glucocorticoids: behavioral and pharmacological perspectives. Neuroscience & Biobehavioral Reviews. 23, 337-344.

Haller, J. and Kruk, M. R., 2006. Normal and abnormal aggression: human disorders and novel laboratory models. Neuroscience & Biobehavioral Reviews. 30, 292-303.

Haller, J., Millar, S. and Kruk, M. R., 1998b. Mineralocorticoid Receptor Blockade Inhibits Aggressive Behaviour in Male Rats. Stress. 2, 201-207.

Haller, J., Millar, S., van de Schraaf, J., de Kloet, R. E. and Kruk, M. R., 2000. The Active Phase-Related Increase in Corticosterone and Aggression are Linked. Journal of Neuroendocrinology. 12, 431-436.

Heistermann, M., Tari, S. and Hodges, J. K., 1993. Measurement of faecal steroids for monitoring ovarian function in New World primates, Callitrichidae. J. Reprod. Fertil. 99, 243-251.

Hesterman, E. R., Goodrich, B. S. and Mykytowycz, R., 1976. Behavioral and cardiac responses of the rabbit, *Oryctolagus cuniculus*, to chemical fractions from anal gland. J. Chem. Ecol. 2, 25-37.

Hesterman, E. R., Goodrich, B. S. and Mykytowycz, R., 1981. Effect of volatiles collected above fecal pellets on behavior of the rabbit, *Oryctolagus cuniculus*, tested in an experimental chamber. J. Chem. Ecol. 7, 799-815.

Hesterman, E. R. and Mykytowycz, R., 1968. Some observations on the odours of anal gland secretions from the rabbit, Oryctolagus cuniculus (L.). CSIRO Wild. Res. 13, 71-81.

Hutchinson, G. E., 1948. Circular Causal Systems in Ecology. Annals of the New York Academy of Sciences. 50, 221-246.

Iborra, O. and Lumaret, J. P., 1997. Validity limits of the pellet group counts in wild rabbit (*Oryctolagus cuniculus*). Mammalia. 61, 205-218.

Joëls, M., Karst, H., DeRijk, R. and de Kloet, E. R., 2008. The coming out of the brain mineralocorticoid receptor. TRENDS in Neurosciences. 31, 1-7.

Jordan, N. R., Cherry, M. I. and Manser, M. B., 2007. Latrine distribution and patterns of use by wild meerkats: implications for territory and mate defence. Animal Behaviour. 73, 613-622.

Kim, J. J. and Diamond, D. M., 2002. The stressed hippocampus, synaptic plasticity and lost memories. Nature Reviews. Neuroscience. 3, 453-462.

King, J. A., 1973. The Ecology of Aggressive Behavior. Annual review of ecology and systematics. 4, 117-138.

Kneitel, J. M. and Chase, J. M., 2004. Trade-offs in community ecology: Linking spatial scales and species coexistence. Ecology Letters. 7, 69-80.

Koolhaas, J. M., Meerlo, P., De Boer, S. F., Struebbe, J. H. and Bohus, B., 1997. The temporal dynamics of the stress response. Neuroscience and Biobehavioral Reviews. 21, 775-782.

Kruk, M. R., Halász, J., Meelis, W. and Haller, J., 2004. Fast positive feedback between the adrenocortical stress response and a brain mechanism involved in aggressive behavior. Behavioral Neuroscience 118, 1062-1070.

Lack, D. L., 1954. The natural regulation of animal numbers. Clarendon Press (Oxford)

Lees, A. C. and Bell, D. J., 2008. A conservation paradox for the 21st century: the European wild rabbit Oryctolagus cuniculus, an invasive alien and an endangered native species. Mammal Review. 38, 304-320.

Letty, J., Aubineau, J. and Marchandeau, S., 2008. Improving rabbit restocking success: a review of field experiments in France. In: Alves PC, Ferrand N and Hackländer K (ed) Lagomorph biology: evolution, ecology and conservation. Springer-Verlag, Berlin.

Letty, J., Marchandeau, S., Clobert, J. and Aubineau, J., 2000. Improving translocation success: an experimental study of antistress treatment and release method for wild rabbits. Animal Conservation. 3, 211-219.

Letty, J., Marchandeau, S., Reitz, F., Clobert, J. and Sarrazin, F., 2002. Survival and movements of translocated wild rabbits (*Oryctolagus cuniculus*). Game and Wildlife Science. 19, 1-23.

Li, C., Jiang, Z., Tang, S. and Zeng, Y., 2007. Influence of enclosure size and animal density on fecal cortisol concentration and aggression in Pe`re David's deer stags. General and Comparative Endocrinology 151, 202–209.

Lockley, R. M., 1961. Social structure and stress in the rabbit warren. Journal of Animal Ecology. 30, 385-423.

López-Bao, J. V., Rodríguez, A. and Palomares, F., 2008. Behavioural response of a trophic specialist, the Iberian lynx, to supplementary food: Patterns of food use and implications for conservation. Biological Conservation. 141, 1857–1867.

López-López, P., García-Ripollés, C., Aguilar, J. M., García-López, F. and Verdejo, J., 2006. Modelling breeding habitat preferences of Bonelli´s eagle (Hieraaetus fasciatus) in relation to topography, disturbance, climate and land use at different spatial scales. . Journal of Ornithology. 147, 97-106.

López-Martínez, N., 2008a. The Lagomorph fossil record and the origin of the European rabbit. In: Alves, P. C., Ferrand, N. and Hackländer, K., Lagomorph Biology: Evolution, Ecology and Conservation. Springer-Verlag, pp. 27-46.

López-Martínez, N., 2008b. The Lagomorph Fossil Record and the Origin of the European Rabbit. In: Alves, P. C., Ferrand, N. and Hackländer, K., Lagomorph biology: evolution, ecology and conservation. Springer-Verlag Berlin, pp. 27-46.

MacDougall-Shackleton, S. A., Dindia, L., Newman, A. E. M., Potvin, D. A., Stewart, K. A. and MacDougall-Shackleton, E. A., 2009. Stress, song and survival in sparrows. Biology Letters 5, 746-748.

Maher, C. R. and Lott, D. F., 1995. Definitions of territoriality used in the study of variation in vertebrate spacing systems. Animal Behaviour. 49, 1581-1597.

Maher, C. R. and Lott, D. F., 2000. A Review of Ecological Determinants of Territoriality within Vertebrate Species. The American Midland Naturalist. 143, 1-29.

Makara, G. B. and Haller, J., 2001. Non-genomic effects of glucocorticoids in the neural system: Evidence, mechanisms and implications. Progress in Neurobiology. 65, 367-390.

Mark, H., 2001. Qualitative Discriminant Analysis. In: Burns DA and Ciurczak EW (ed) Handbook of Near-Infrared Analysis. Marcel Dekker, Inc., New York.

Martins, H., Milne, J. A. and Rego, F., 2002. Seasonal and spatial variation in the diet of the wild rabbit (*Oryctolagus cuniculus* L.) in Portugal. J. Zool. 258, 395-404.

Massé, A. and Côté, S. D., 2012. Linking habitat heterogeneity to space use by large herbivores at multiple scales: From habitat mosaics to forest canopy openings. Forest Ecology and Management. 285, 67-76.

McKittrick, C. R., Blanchard, D. C., Hardy, M. P. and Blanchard, R. J., 2009. Social Stress Effects on Hormones, Brain, and Behavior. In: Inc., E., Hormones, Brain and Behavior. pp. 341-373.

Mentaberre, G., López-Olvera, J. R., Casas-Díaz, E., Marco, I. and Lavín, S., 2010. Haloperidol and azaperone in drive-net captured southern chamois (Rupicapra pyrenaica) Journal of Wildlife Diseases. 46, 923-928.

Mikics, E., Barsy, B. and Haller, J., 2007. The effect glucocorticoids on aggressiveness in established colonies of rats. Psychoneuroendocrinology 32, 160-170.

Mikics, E., Kruk, M. R. and Haller, J., 2004. Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats. Psychoneuroendocrinology. 29, 618-635.

Mock, D. W. and Ploger, B. J., 1987. Parental manipulation of optimal hatch asynehrony in cattle egrets: an experimental study. Animal Behaviour. 35, 150-160.

Monclús, R. and De Miguel, F. J., 2003. Distribución espacial de las letrinas de conejo (*Oryctolagus cuniculus*) en el monte de Valdelatas (Madrid). Galemys. 15, 157-165.

Monclús, R., Palomares, F., Tablado, Z., Martínez-Fontúrbel, A. and Palme, R., 2009. Testing the threat-sensitive predator avoidance hypothesis: physiological responses and predator pressure in wild rabbits. Oecologia 158, 615–623.

Monclús, R., Rödel, H. G., Palme, R., Von Holst, D. and De Miguel, F. J., 2006a. Non-invasive measurement of the physiological stress response of wild rabbits to the odour of a predator. Chemoecology. 16, 25-29.

Monclús, R., Rödel, H. G. and Von Holst, D., 2006b. Fox Odour Increases Vigilance in European Rabbits: A Study under Semi-Natural Conditions. Ethology. 112, 1186–1193.

Monclús, R., Rödel, H. G., Von Holst, D. and De Miguel, J., 2005. Behavioural and physiological responses of naïve European rabbits to predator odour. Animal Behaviour. 70, 753-761.

Moreno, S., Beltran, J. F., Cotilla, I., Kuffner, B., Laffite, R., Jordán, G., Ayala, J., Quintero, C., Jiménez, A., Castro, F., Cabezas, S. and Villafuerte, R., 2007. Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain. Wildlife Research. 34, 652-658.

Moreno, S., Villafuerte, R., Cabezas, S. and Lombardi, L., 2004. Wild rabbit restocking for predator conservation in Spain. Biological Conservation. 118, 183-193.

Moynihan, J. A. and Ader, R., 1996. Psychoneuroimmunology: Animal Models of Disease. Psychosomatic Medicine 58, 546-558.

Muñoz, G., 1960. Anverso y reverso de la mixomatosis. Dirección General de Montes, Madrid.

Myers, K. and Poole, W. E., 1959. A study of the biology of the Wild Rabbit, Oryctolagus cuniculus (L) in confined populations. I. The effects of density on home range and the formation of breeding groups. C.S.I.R.O. Wildlife Research 4, 14-27.

Myers, K. and Poole, W. E., 1962. A study of the biology of the Wild Rabbt, Oryctolagus cuniculus (L) in confined populations. III. Reproduction. Australian Journal of Zoology. 10, 225-267.

Myers, K. and Poole, W. E., 1963. A study of the biology of the wild rabbit, Oryctolagus cuniculus (L.), in confined populations. V. Population dynamics. CSIRO Wildlife Research. 8, 166-203.

Mykytowycz, R., 1958. Social behaviour of an experimental colony of wild rabbits, Oryctolagus cuniculus (L.) I. Establishment of the colony. CSIRO Wildlife Research. 3, 7-25.

Mykytowycz, R., 1959a. Social behaviour of an experimental colony of wild rabbits, Oryctolagus cuniculus (L.). II. First breeding season. Wildlife Survey Section, CSIRO. 1-13.

Mykytowycz, R., 1959b. Social behaviour of an experimental colony of wild rabbits, Oryctolagus cuniculus (L.). III. Second breeding season. Wildlife Survey Section, CSIRO. 1-20.

Mykytowycz, R., 1962. Territorial function of chin gland secretion in the rabbit, Oryctolagus cuniculus (L.). Nature. 193, 799.

Mykytowycz, R., 1964. Territorial marking by rabbits. Scientific American. 218, 116-126.

Mykytowycz, R., 1966. Observations on odoriferous and other glands in the Australian wild rabbit, Oryctolagus cuniculus (L.), and the hare, Lepus europaeus P. II. The inguinal glands. Wildlife Research. 11,

Mykytowycz, R. and Gambale, S., 1965. A study of inter-warren activities and dispersal of wild rabbits, *Oryctolagus cuniculus* (L.), living in a 45-ac paddock. Wildlife Research. 10, 111-123.

Mykytowycz, R. and Gambale, S., 1969. The distribution of dung-hills and the behaviour of free-living rabbits, Oryctolagus cuniculus (L.), on them. Forma et Functio. 1, 333-349.

Mykytowycz, R. and Goodrich, B. S., 1974. Skin glands as organs of communication in mammals. The Journal of Investigative Dermatology. 62, 124-131.

Mykytowycz, R. and Hesterman, E. R., 1970. The behaviour of captive wild rabbits, Oryctolagus cuniculus (L.) in response to strange dung-hills. Forma et Functio. 2, 1-12.

Mykytowycz, R., Hesterman, E. R., Gambale, S. and Dudzinski, M. L., 1976. A comparison of the effectiveness of the odors of rabbits, *Oryctolagus cuniculus*, in enhancing territorial confidence. Journal of Chemical Ecology. 2, 13-24.

Navidi, W., 2008. Statistics for engineers and sciencists. McGraw-Hill,

Norris, K. H. and Williams, P. C., 1984. Optimization of mathematical treatments of raw Near-Infrared signal in the measurement of protein in hard red spring wheat. I. Influence of particle size. Cereal Chem. 61, 158-165.

Oitzl, M. S., Fluttert, M. and De Kloet, E. R., 1994. The effect of corticosterone on reactivity to spatial novelty is mediated by central mineralocorticosteroid receptors. European Journal of Neuroscience. 6, 1072-1079.

Osborne, B. G., Mertens, B., Thompson, M. and Fearn, T., 1993. The authentication of Basmati rice using near infrared spectroscopy. J. Near Infrared Spectrosc. 1, 77-83.

Pasikatan, M. C., Steele, J. L., Spillman, C. K. and Haque, E., 2001. Near infrared reflectance spectroscopy for online particle size analysis of powders and ground materials. J. Near Infrared Spectrosc. 9, 153-164.

Pech, R. P., Sinclair, A. R. E., Newsome, A. E. and Catling, P. C., 1992. Limits to predator regulation of rabbits in Australia: evidence from predator-removal experiments. Oecologia. 89, 102-112.

Perreault, W. D. and Leigh, L. E., 1989. Reliability of nominal data based on qualitative judgements. J. Marketing Res. 26, 135-148.

Pinheiro, J. C. and Bates, D. M., 2000. Mixed-Effects Models in S and S-PLUS. Springer-Verlag, New York.

Potegal, M., 1992. Time course of aggressive arousal in female hamsters and male rats. Behavioral and Neural Biology 58, 120-124.

Putman, R. J., 1984. Facts from faeces. Mammal Rev. 14, 79-97.

Ralls, K., 1971. Mammalian scent marking. Science. 171, 443-449.

Ramdohr, S., Bornemann, H., Plotz, J. and Bester, M. N., 2001. Immobilization of free-ranging adult male southern elephant seals with Immobilon(tm) (etorphine/acepromacine) and ketamine. South African Journal of Wildlife Research. 31, 135-140.

Ramsay, D. and Lewis, M., 2003. Reactivity and Regulation in Cortisol and Behavioral Responses to Stress. Child Development. 74, 456–464.

Redpath, S. M. and Thirgood, S. J., 1997. Birds of prey and red grouse. Stationery Office, London.

Rödel, H. G., Bora, A., Kaetzke, P., Khaschei, M., Hutzelmeyer, H. and Von Holst, D., 2004a. Over-winter survival in subadult European rabbits: weather effects, density dependence, and the impact of individual characteristics. Oecologia. 140, 566-576.

Rödel, H. G., Bora, A., Kaiser, J., Kaetzke, P., Khaschei, M. and Von Holst, D., 2004b. Density-dependent reproduction in the European rabbit: a consequence of individual response and age-dependent reproductive performance. Oikos. 104, 529-539.

Rödel, H. G., Monclús, R. and von Holst, D., 2006. Behavioral styles in European rabbits: Social interactions and responses to experimental stressors. Physiology and behavior. 89, 180-188.

Rogovin, K., Randall, J. A., Kolosova, I. and Moshkin, M., 2003. Social correlates of stress in adult males of the great gerbil, Rhombomys opimus, in years of high and low population densities. Hormones and Behavior 43, 132-139.

Rolland, R. M., Hunt, K. E., Kraus, S. D. and Wasser, S. K., 2005. Assessing reproductive status of right whales /Eubalaena glacialis) using fecal hormone metabolites. Gen. Comp. Endocrinol. 142, 308-317.

Romero, L. M., 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution. 19, 249-255.

Romero, L. M. and Wikelski, M., 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proceedings of the Royal Society Series B. 277, 3157-3162

Rouco, C., 2008. Restauración de las poblaciones de conejo de monte y mejora de la gestión para su conservación. Universidad de Castilla-La Mancha,

Rouco, C., Ferreras, P., Castro, F. and Villafuerte, R., 2008. Effect of terrestrial predator exclusion on short term survival of translocated European wild rabbits. Wildlife Research.

Rouco, C., Ferreras, P., Castro, F. and Villafuerte, R., 2010. A longer confinement period favors European wild rabbit (Oryctolagus cuniculus) survival during soft releases in low-cover habitats. European Journal of Wildlife Research. 56, 215–219.

Rouco, C., Villafuerte, R., Castro, F. and Ferreras, P., 2011. Responses of naïve and experienced European rabbits to predator odour. European Journal of Wildlife Research 57, 395-398.

Sadée, W., Abshagen, U., Finn, C. and Rietbrock, N., 1974. Conversion of spironolactone to canrenone and disposition kinetics of spironolactone and canrenoate-potassium in rats. Naunyn-Schmiedeberg's Archives of Pharmacology 283, 303-318.

Salamon, M. and Davies, N. W., 1998. Identification and variation of volatile compounds in sternal gland secretions of male koalas (Phascolarctos cinereus). J. Chem. Ecol. 24, 1659-1676.

Sapolsky, R. M., 1994. Why zebras don’t get ulcers: A guide to stress, stress-related diseases, and coping New York.

Sapolsky, R. M., Krey, L. C. and McEwen, B., 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocrine Reviews. 7, 284-301.

Sapolsky, R. M., Romero, L. M. and Munck, A. U., 2000. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. Endocrine Reviews 21, 55-89.

Schacter, D. L. and Buckner, R. L., 1998. Priming and the Brain. Neuron. 20, 185-195.

Schalken, A. P. M., 1976. Three types of pheromones in the domestic rabbit, Oryctolagus cuniculus (L.). Chem. Sens. 2, 139-155.

Selye, H., 1950. Stress and the general adaptation syndrome. British medical Journal. 1, 1387–1392.

Shenk, J. S. and Westerhaus, M. O., 1995. Routine operation, calibration, development and network system management manual. NIRSystems, Inc., 12101 Tech Road, Silver Spring

Shenk, J. S. and Westerhaus, M. O., 1996. Near Infrared Spectroscopy: The Future Waves. . NIR Publications, Chichester, West Sussex, UK,

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R. and Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia. 166, 869-887.

Skibsted, E. T. S., Boelens, H. F. M., Westerhuis, J. A., Witte, D. T. and Smilde, A. K., 2004. New indicator for optimal preprocessing and wavelength selection of near-infrared spectra. Appl. Spectrosc. 58, 264-271.

Smith, G. C., Prickett, A. J. and Cowan, D. P., 2007. Costs and benefits of rabbit control options at the local level. International Journal of Pest Management. 53, 317-321.

Sneddon, I. A., 1991. Latrine use by the European rabbit (*Oryctolagus cuniculus*). J. Mammal. 72, 769-775.

Sousa, N., Cerqueira, J. J. and Almeida, O. F. X., 2008. Corticosteroid receptors and neuroplasticity. Brain Research Reviews. 57, 561-570.

Southern, H. N., 1940. Coprophagy in the Wild Rabbit. Nature. 145, 262.

Speakman, J. R., 2008. The physiological costs of reproduction in small mammals. Philosophical Transactions of the Royal Society B 363, 375–398.

Spiegelman, C. H., McShane, M. J., Goetz, M. J., Motamedi, M., Yue, Q. L. and Coté, G. L., 1998. Theoretical justification of wavelength selection in PLS calibration: development of a new algorithm. Anal. Chem. 70, 35-44.

Summers, C. H. and Winberg, S., 2006. Interactions between the neural regulation of stress and aggression. The Journal of Experimental Biology. 209, 4581-4589.

Sun, L. and Müller-Schwarze, D., 1998. Anal gland secretion codes for family membership in the beaver. Behav. Ecol. Sociobiol. 44, 199-208.

Taché, Y., Martínez, V., Million, M. and Wang, L., 2001. Stress and the Gastrointestinal Tract III. Stress-related alterations of gut motor function: role of brain

corticotropin-releasing factor receptors. American Journal of Physiology: Gastrointestinal and Liver Physiology. 280, G173-G177.

Taylor, R. H. and Williams, R. M., 1956. The use of pellet counts for estimating the density of populations of the wild rabbit, *Oryctolagus cuniculus* (L.). N.Z.J. Sci. Technol. Sect. 38, 236-256.

Teixeira, C. P., de Azevedo, C. S., Mendl, M., Cipreste, C. F. and Young, R. J., 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. Animal Behaviour. 73, 1-13.

Tolleson, D. R., Randel, R. D., Stuth, J. W. and Neuendorff, D. A., 2005. Determination of sex and species in red and fallow deer by near infrared reflectance spectroscopy of the faeces. Small Rumiant Res. 57, 141-150.

Tolleson, D. R., Teel, P. D., Stuth, J. W., Strey, O. F., Jr., T. H. W. and Carstens, G. E., 2007. Fecal NIRS: Detection of tick infestations in cattle and horses. Vet. Parasitol. 144, 146-152.

Touma, C., Palme, R. and Sachser, N., 2004. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Hormones and Behavior. 45, 10-22.

Touma, C., Sachser, N., Möstl, E. and Palme, R., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. General and Comparative Endocrinology. 130, 267-278.

Trout, R. C. and Smith, G. C., 1998. Long-term study of litter size in relation to population size in rabbits (*Oryctolagus cuniculus*) in Lincolnshire, England. Journal of Zoology. 246, 347-350.

Trout, R. C. and Tittensor, A. M., 1989. Can predators regulate wild rabbit *Oryctolagus cuniculus* population density in England and Wales? Mammal Review. 19, 153-173.

Turchin, P., 1995. Population regulation: old arguments and a new synthesis. In: Cappuccino, N. and Price, P. W., Population dynamics new approaches and synthesis. Chapman & Hall, pp. 19–40.

Turchin, P., 1999. Population Regulation: A Synthetic View. Oikos. 84, 153-159

Turin, L., 1996. A Spectroscopic Mechanism for Primary Olfactory Reception. Chem. Sens. 21, 773-791.

van der Vegt, B. J., Lieuwes, N., van de Wall, E. H. E. M., Moya-Albiol, L., Martínez-Sanchis, S., Kato, K., de Boer, S. and Koolhaas, J. M., 2003. Activation of serotonergic neurotransmission during the performance of aggressive behavior in rats. Behavioral Neuroscience. 117, 667-674.

Vargas, J. M., Farfán, M. A., Guerrero, J. C., Barbosa, A. M. and Real, R., 2007. Geographical and environmental correlates of big and small game in Andalusia (southern Spain). Wildlife Research. 34,

Villafuerte, R. and Delibes-Mateos, M., 2007. El conejo. In: Palomo, L. J., Gisbert, J. and Blanco, J. C., Atlas y Libro Rojo de los Mamíferos Terrestres de España. Ministerio de Medioambiente-Dirección General para la Biodiversidad-SECEM, Madrid, pp. 490–491.

Virgós, E., Cabezas-Díaz, S., Malo, A., Lozano, J. and López-Huertas, D., 2003. Factors shaping European rabbit abundance in continuous and fragmentated populations of central Spain. Acta Theriologica. 48, 113-122.

Von Holst, D., 1998. The concept of stress and its relevance for animal behavior. Advances in the Study of Behavior. 27, 1-131.

Von Holst, D., Hutzelmeyer, H., Kaetzke, P., Khaschei, M., Rödel, H. G. and Schrutka, H., 2002. Social rank, fecundity and lifetime reproductive success in wild European rabbits (*Oryctolagus cuniculus*). Behavioural Ecology and Sociobiology. 51, 245-254.

Von Holst, D., Hutzelmeyer, H., Kaetzke, P., Khaschei, M. and Schönheiter, R., 1999. Social Rank, Stress, Fitness, and Life Expectancy in Wild Rabbits. Naturwissenschaften. 86, 388-393.

Wikelski, M. and Cooke, S. J., 2006. Conservation physiology. Trends in Ecology and Evolution. 21, 38-46.

Williams, D., Acevedo, P., Gortazar, C., Escudero, M. A., Labarta, J. L., Marco, J. and Villafuerte, R., 2007. Hunting for answers: rabbit (*Oryctolagus cuniculus*) population trends in northeastern Spain. European Journal of Wildlife Research. 53, 19-28.

Wingfield, J. C., Maney, D. L., Breuner, C. W., Jacobs, J. D., Lynn, S., Ramenofsky, M. and Richardson, R. D., 1998. Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage". American Zoologist. 38, 191-206.

Wood, D. H., 1980. The demography of a rabbit population in an arid region of New South Wales Australia. J. Anim. Ecol. 49, 55-79.

Workman, J., 2001. Interpretive spectroscopy for near-infrared. In: Handbook of Organic Compounds. Academic Press: San Diego, CA,

Workman Jr., J. J., 2001. NIR Spectroscopy Calibration Basics. In: Burns DA and Ciurczak EW (ed) Handbook of Near-Infrared Analysis.
. Marcel Dekker, Inc., New York.

Worton, B. J., 1989. Kernel methods for estimating the utilization distribution in home-range studies. Ecology. 70, 164-168.

Wynne-Edwards, V. C., 1959. The control of population density through social behavior: A hypothesis. Ibis. 101, 436-441.

Zhang, J. X., Sun, L., Zhang, Z. B., Wang, Z. W., Chen, Y. and Wang, R., 2002. Volatile compounds in anal gland of Siberian weasels (Mustela sibirica) and steppe polecats (M. eversmanni). J. Chem. Ecol. 28, 1287-1297.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. and Smith, G. M., 2009. Mixed Effects Models and Extensions in Ecology with R. Springer, New York.

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TÍTULO DE LA TESIS: Study of territorial behaviour and stress for the improvement of European wild rabbit restocking programs.

DOCTORANDO/A: Leire Ruiz Aizpurua

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda, Leire Ruiz Aizpurúa, ha desarrollado durante cuatro años una labor investigadora satisfactoria bajo mi dirección. Durante este tiempo la doctoranda ha ido cumpliendo con los objetivos previstos, tanto en lo que refiere al trabajo de campo como en la elaboración de los artículos científicos que le dan cuerpo a su tesis doctoral. Para ello ha realizado las labores de búsqueda bibliográfica, planteamiento de hipótesis de trabajo y enfoque adecuado de los resultados de campo. Como resultado la información que ha ido adquiriendo durante el trabajo de campo ha sido organizada adecuadamente en formato de artículos científicos que se hallan en fase de revisión.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, __30__ de __enero_____ de ___2013_____

Firma del/de los director/es

Fdo.:__Francisco Sánchez Tortosa