Migratory strategies of the lesser kestrel:

determining wintering areas and condition for migration

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Airam Rodríguez Martín
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Estrategias migratorias del cernícalo primilla: determinación de las áreas de invernada y de la condición para la migración

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PhD Thesis

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A mi familia

"So much trouble in the world"
Robert N. Marley

"Life should be simpler"
Gary R. Bortolotti

"Pásenlo bien, pásenlo bien"
Domingo (*Cabrera*) Rodríguez del Rosario (*Chirivitas*)

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Preface

In 2006, I enjoyed an I3P postgraduate fellowship at the Island Ecology and Evolution Research Group (Instituto de Productos Naturales y Agrobiología, CSIC) under the supervision of Drs. Manuel Nogales and Alfredo Valido. There, I was introduced to the biological sciences aiming at revealing the endozoochoric seed dispersal patterns of the threatened native temperate forest of Canary Islands. After that, I sent tens of e-mails to several Spanish researchers asking for supervision on raptors or petrels studies. Finally, I applied in 2007 for an I3P pre-doctoral fellowship (4 years) at the Department of Evolutionary Ecology of the Estación Biológica de Doñana, CSIC, under the supervision of Drs. Juan J. Negro and Miguel Alcaide. I was lucky and grant was awarded to me to study phenotypic and genotypic indices of individual quality in the lesser kestrel.

The lesser kestrel has been classified as vulnerable. This fact precluded us from carrying out experiments on wild birds. Despite the Lesser kestrel is one of the most studied species in the world (more than 200 scientific papers published, several popular scientific monographs and at least, 8 PhD thesis defended only in Spain), the level of knowledge on the wintering areas of the lesser kestrel was scanty before this study. It was indeed known that lesser kestrel wintered both in the Sahel region and in South Africa, but the origin of populations remained unknown. On the other hand, more information on the wintering ecology, migration routes, and the physiological implications of such behaviour is urgently needed for the appropriate management of the threatened lesser kestrel in Africa. This lack of knowledge and the difficulty to carry out experiments were decisive in the restructuration and reorganization of my PhD. For this reason, the delivery date of this study has been prolonged up to October 2011.

My PhD thesis deals with an array of topics within the fields of ecology, genetics and physiology. Here, we use extrinsic (geolocators) and intrinsic (MHC genes) markers to study migration and wintering areas, and plasma blood metabolites to study nutritional condition. Furthermore, I present two technical notes: one deals with the effect of geolocators on breeding parameters; and another details a procedure to accurately genotype MHC genes. The fieldwork in connection with my PhD was mainly conducted at two breeding colonies in the Province of Huelva (Southern Spain) during 2007 to 2009. Even though I also present information from the lesser kestrel wintering areas,

unfortunately I did not travel to South Africa or Senegal looking for lesser kestrel feathers at the roosts.

During the period (2007-2011), I have been located in Seville at EBD-CSIC, although I enjoyed two research stays to complete my training: one in Finland under the supervision of Erkki Korpimäki (University of Turku) during August-October 2008 and another on Kauai, Hawaiian Archipelago, under the supervision of Andrea Erichsen and Nick D. Holmes (Kauai Seabird Habitat Conservation Program, Hawaii State Department of land and Natural Resources, University of Hawaii) during October-November 2010. During the last years, and thus concurrent my PhD work, I have helped to produce several other scientific papers and assisted to five international congresses; some using lesser kestrels as models, but also related to other bird species or even topics far away form those covered in my PhD thesis. These investigations may have diverted my efforts from the core of my thesis, but I wish to think they have also contributed in some way to my training as a field ecologist and conservationist.

Resumen

Estrategias migratorias del cernícalo primilla: determinación de las áreas de invernada y de la condición para la migración.

A pesar de que el cernícalo primilla *Falco naumanni* es una de las aves más estudiadas en el campo, poco se sabe de las áreas de invernada de las poblaciones reproductoras y de su condición nutricional. El principal objetivo de la presente tesis es aportar información básica sobre la conectividad migratoria de la especie entre las áreas de cría y de invernada. Nosotros usamos, por primera vez en rapaces, geolocalizadores para determinar las áreas de invernada de una población occidental de su rango de cría. Encontramos que los cernícalos invernaron en las inmediaciones de los ríos senegaleses Senegal y Saloum. Basándonos en datos de longitud, inferimos que la duración de la migración post-nupcial es 5 ± 1 días, mientras que la de la migración pre-nupcial es más larga y variable (24 ± 10 días). Los geolocalizadores no mostraron efectos perjudiciales en los adultos marcados (excepto un arnés mal fijado). La tasa de retorno fue similar a la observada en otros años y colonias (15 retornaron en 2008 de los 20 marcados en 2007); y el peso de individuos marcados no difirió de los no-marcados en el momento de la recaptura. Los parámetros de cría (tamaño de puesta y número de pollos volanderos) no fueron afectados por los geolocalizadores. Sin embargo, una mayor mortalidad de pollos de las parejas marcadas fue observada en 2008, así como un aumento en el nivel de triglicéridos y ácido úrico en el plasma de los pollos con al menos un padre marcado.

Por otra parte, usamos una aproximación no invasiva basada en un marcador genético, el complejo principal de histocompatibilidad (MHC, siglas en inglés), para inferir el origen de los individuos que invernan en las áreas más importantes para el cernícalo primilla. Para ello, aprovechamos la información disponible sobre la estructuración de los genes del MHC de clase II B en el área de cría y colectamos plumas mudadas bajo grandes dormideros localizados en Senegal y Sudáfrica. Las puntas y los coágulos de sangre de dichas plumas fueron usadas como fuente de DNA, a partir del cual amplificamos por PCR el segundo exón de dichos genes. Alelos privados del área de cría occidental fueron encontrados mayoritariamente en Senegal, indicando una fuerte conectividad entre estas dos áreas. Los cernícalos invernantes en Sudáfrica fueron genéticamente distintos a los

europeos, lo que sugiere que los dormideros sudafricanos están compuestos por individuos asiáticos. Esto demuestra que al menos a escala continental, los genes del MHC pueden servir como marcadores intrínsecos para el estudio de la migración de aves.

Aunque los genes del MHC son muy populares entre los biólogos evolutivos por su rol en el sistema inmunitario y selección sexual, su genotipaje permanece aún desafiante a pesar de los importantes avances que se están alcanzando en la actualidad. Nosotros testamos el rendimiento del algoritmo bayesiano PHASE implementado en el programa informático DNAsp para la inferencia de haplotipos de estos genes, los cuales presentan características que han sido descritas como fuente de inexactitudes (alta heterozigosidad, gran variación genética, mezcla de poblaciones). Para este fin, aprovechamos una gran base de datos de genotipos conocidos a través de técnicas tradicionales (clonaje y herencia de alelos de padres a hijos) de ambos loci (MHC de clase I y II). En ambos loci, encontramos que el ratio alelo/individuo debe estar en torno a 1:2 para obtener un 100% de exactitud en la salida de PHASE.

Finalmente, evaluamos las fuentes de variación de la condición nutricional de cernícalos adultos y pollos en su área de cría a través de los niveles de los siguientes metabolitos plasmáticos (triglicéridos, colesterol, ácido úrico y urea). En general, encontramos que los cernícalos presentaron consistentemente valores más altos de triglicéridos, ácido úrico y urea (los dos últimos, ocasionalmente) que otras rapaces. Esto fue interpretado como una consecuencia de la mayor frecuencia de alimentación y de la dieta eminentemente insectívora del cernícalo primilla. Casi todos los factores explorados (año, colonia, hora de muestreo, peso, fecha de puesta y fecha de captura) influenciaron al menos un parámetro bioquímico. El más influyente fue la hora de muestreo, el cual alcanzó significación para todos los parámetros en pollos, mientras que para adultos tan sólo en ácido úrico y urea. Los valores bioquímicos de los pollos incrementaron a lo largo de la mañana como consecuencia del ayuno de la noche, es decir, por la ingesta de comida a lo largo de la mañana. El incremento en la carga de trabajo durante la mañana (cebas de cortejo y de alimentación de pollos) podría explicar el aumento de ácido úrico y urea en adultos.

En esta tesis, se empleó geolocalizadores y genes del MHC para el estudio de la migración. Ambas metodologías fueron usadas por primera vez en rapaces y en aves, respectivamente, y han aportado información básica y útil para la conservación del cernícalo primilla. Después del presente estudio, parece claro que las poblaciones reproductoras occidentales y orientales invernan en áreas diferentes. Así los cernícalos europeos invernan en el Sahel, mientras que los asiáticos lo hacen en el sur de África.

Además, hemos demostrado que los metabolitos plasmáticos de los cernícalos difieren con respecto al de otras rapaces, posiblemente como consecuencia de sus hábitos alimenticios y de caza.

Introduction

Generalities on avian migration

Defining migration is a difficult task partially because of the huge variability of animal movements. A generally accepted definition among students of avian migration is the regular, endogenously controlled, seasonal movements between breeding and non-breeding areas (Salewski & Bruderer 2007). Despite that there is no consensus on the precise definition of avian migration, it is widely agreed that is one of the most impressive natural phenomena in the world. Annually billions of birds travel to their breeding and non-breeding grounds, stopping to eat and rest, twice each year worldwide. These journeys can be as much as 80000 kilometres long in the case of the Arctic tern *Sterna paradisaea* (Egevang *et al.* 2010) and reached in non-stop flights (Gill *et al.* 2009, Klaassen *et al.* 2011). As a consequence of the biased distribution of land masses on the Earth surface, most birds have a North-South direction during their post-nuptial migrations, and spend their annual non-breeding period at lower latitudes than their breeding period or at similar latitudes, but in the opposite hemisphere (Newton 2008).

Markers for migration studies

Technological advances to study migration emerged a long time ago, including tracking devices such as radar. However, an exponential increase has taking place during the last two decades. Our capacity to trace or follow individual birds is mainly limited by bird size, economic costs and probability of recapture. Extrinsic markers are tagged to birds. Individual identification, by means of leg bands, neck collars, patagial tags or plumage markings, rank among the most widely used markers. During the last century, ringing has been vital for elucidating patterns of migration, and it has provided the bulk of information on this topic. Nevertheless, in many cases only a small proportion (sometimes no birds) is ever recovered or re-sighted. Remote-sensing capability represents a technological advance over such mark-recapture techniques. Radio tracking is a relative unexpensive method to track individuals, but researcher needs to follow the birds within a minimal distance to

reach the signal of tagged transmitters. Satellite transmitters let researchers to follow the birds without even leaving office, as position data from the transmitters are recovered through satellites (i.e. no recapture is needed). However, this tool is currently unachievable to most researchers because of its high economic costs, and too heavy for the most bird species (the lightest Platform Transmitter Terminal's –PTT– on sell is now on five grams). The new miniaturized geolocators (as light as 1 gram) require of recapture to download the data, they only report two locations per day and their accuracy is quite lower than PTTs. The main advantages are their low economic cost and weight, opening their use to a huge number of small-sized species (Stutchbury *et al.* 2009).

Between the intrinsic markers, the most useful to study bird migration are stable isotopes, genetic markers and trace elements (Coiffait *et al.* 2009). The proportions of different stable isotopes of naturally occurring elements such as carbon, nitrogen and hydrogen vary across the environment in systematic ways. Thanks to strong latitudinal gradients in the isotopic ratio of hydrogen, isotopic analyses of different tissues, such as blood, feathers or claws have permitted researchers to identify the areas where renovation or growth of those tissues took place. However, these markers only provide information on the areas where tissues grew. Furthermore, latitudinal gradients are not always so clear. For example, stable hydrogen isotope ratios in precipitation show a continent-wide pattern in North America, but the European pattern is not so apparent (see Hobson & Wassenaar 2008 for more details).

Genetic markers are useful to study migration, but only if genetic variation of populations is geographically structured (Wink 2006). Thus, individuals sampled at any place of migration can be assigned back to their most probable geographically structured population (Webster *et al.* 2002). Mitochondrial DNA is the most used genetic marker in birds, especially in the recent past. Microsatellites have become one of the most popular genetic marker due to their pronounced polymorphism and the parallel development of assignment tests such as Structure (Pritchard *et al.* 2000) and others (reviewed in Manel *et al.* 2005). Other genetic markers such as randomly amplified polymorphic DNA (RAPD) or arbitrary fragment length polymorphism (AFLP) provide valuable alternatives to microsatellites and mitochondrial DNA.

Trace elements (also called biogeochemical markers) are chemical markers, analogous to stable isotope ratios. The trace element signatures in tissues are derived from diet. However, this method has been used successfully in only a few studies (see Coiffait *et al.* 2009).

MHC as a marker

The MHC (Major Histocompatibility Complex) constitutes the most important genetic component of the vertebrate immune system. MHC genes encode cell-surface glycoproteins that bind antigens derived from pathogens or parasites. Its primary roles are recognize foreign proteins, present them to specialist immune cells and initiate the appropriate immune response. Genetic variation at MHC genes largely determines the foreign peptides can be recognized by the individual, and how individuals can respond to them. Several evolutionary mechanisms have been suggested to generate and maintain the high levels of genetic polymorphisms commonly found within the antigen-binding sites (reviewed in Sommer 2005, Piertney & Oliver 2006). Given the abundance and virulence of pathogens and parasites geographically vary, the parasite-mediated selection differently act on these genes within host distribution range. Thus, MHC genes may be more structured than other neutral variation markers such as microsatellites, and consequently, MHC may be a useful genetic marker for migration studies.

Conservation of migratory species

Migratory birds differ from residents in that limiting factors of their populations operate on more than one part of their range, i.e. in breeding and non breeding areas as well as migration routes and stopover sites (Newton 2004). As a consequence, European migratory bird species have suffered sustained and more severe population declines than resident species (Sanderson *et al.* 2006). Conserving avian migrants poses major scientific and political challenges (Bowlin *et al.* 2010). First, for the majority of bird species, limited basic life history information on the location and use of wintering areas, time of migration, use of stopovers, or migratory connectivity is available. Second, understanding seasonal interactions between wintering and breeding areas allow us evaluating their influence on population dynamics (Webster *et al.* 2002). Third, synchronicity and phenology of bird migrations is a key topic because they are tied to the emergence of food resources. The effect of climate change on these parameters is unknown for the majority of species (Pulido 2007). Forth, birds have demonstrated to change their migratory behaviours in a short time scale (Nikita *et al.* 2008, Kasper *et al.* 2011). Their flexibility and adaptability to changes in the migratory landscape will determine the degree of affection.

The lesser Kestrel as a model species

The lesser kestrel *Falco naumanni* is one of the smallest European raptor species (along with the Merlin *Falco columbarius* and the Red-Footed Falcon *Falco vespertinus*) (Fergusson-Lees & Christie 2001). Its diet is basically composed of insects (i.e., grasshoppers, beetles, crickets), but it also feeds on small mammals (Rodríguez *et al.* 2010, Pérez-Granados 2010, and references therein; see Fig. 1). It exhibits a significant sexual dimorphism and dichromatism (Fig. 2), and it is associated to steppe and pseudosteppe habitats. It breeds in colonies of up to 100 pairs in towns (e.g., on ruins, churches, castles, buildings), rural areas (barns, abandoned farms, silos) or natural rocky outcrops (Tella *et al.* 1996). It is a monogamous bird, but low levels of extra pair paternity have been detected (Alcaide *et al.* 2005 and references therein). During reproductive duties, both sexes incubate a clutch varying from 3 to 6 eggs. Males provide food to their mates during the laying period and the majority of prey during the early stages of nestlings (Donázar *et al.* 1992).

This migratory falcon's breeding range spans from the Iberian Peninsula through the Mediterranean basin, Asia Minor, Western Asia, to Mongolia and China and its wintering grounds are located in sub-Saharian Africa and South Africa (Rodríguez *et al.* 2009, 2011). In the South of Spain, all juveniles leave the breeding colonies, while approximately 20 % of adult birds are resident (Negro *et al.* 1991).

Its populations have decreased dramatically (c. 95%) in the Western Palearctic since the 1950s, and a reduction of more than 30% of the world population has been estimated, leading to its current Vulnerable status. Habitat degradation and loss, as a result of agriculture intensification, afforestation and urban sprawl in its Western Palearctic breeding grounds, as well as in some winter areas, have been suggested as the main causes of the decline suffered by the species (BirdLife International 2011). As a consequence, numerous breeding programs have been put in place for reintroduction purposes (Pomarol 1993, Alcaide *et al.* 2010).

For more precise information see Cramp & Simmons (1980), Negro (1997) and Ferguson-Lees & Christie (2001).

Fig. 1. Lesser kestrel adult male predating on a locust. Photo: Pepe Antolín

Fig. 2. Lesser kestrel pair (male is perched just on the top of the roof). Photo: Pepe Antolín

Aims

The main aim of this PhD Thesis is to provide basic life history information on migratory connectivity of lesser kestrel populations, decreasing the huge information gap on this topic for this species. The specific aims were:

1) to locate the wintering areas, as well as the migration routes, of Western European Lesser Kestrels by using light level geolocation.
2) to examine the possible effects of geolocators on various breeding parameters (clutch size, number of fledged young, and clutch initiation date) during two consecutive nesting seasons.
3) to evaluate blood biochemistry parameters (triglycerides, cholesterol, urea, and uric acid) of nestlings of geolocator-tagged and control pairs.
4) to test the suitability of MHC markers to infer migratory connectivity between breeding and wintering populations.
5) to test the accuracy and performance of analytical approaches (PHASE algorithm) for the computational inference of the gametic phase across highly polymorphic genes such as those belonging to the MHC.
6) to examine the nutritional condition of lesser kestrels based on selected blood biochemistry parameters.

Thesis outline

In chapter 1, we explain how we fitted, for the first time, geolocators to a bird of prey, the lesser kestrel, to shed light on the wintering areas of the threatened western population.

In chapter 2, we examine the possible effects of geolocators on various breeding parameters (clutch size, number of fledged young, and clutch initiation date) during two consecutive breeding seasons, as well as on the blood biochemistry parameters (triglycerides, cholesterol, urea, and uric acid) of nestlings of geolocator-tagged and control pairs.

In chapter 3, we use, for the first time in birds, MHC loci as genetic markers to study migratory connectivity. This chapter is aimed to unravel the breeding origin of lesser kestrels wintering in two roosts located in Senegal and South Africa.

In chapter 4, we tested the performance of the PHASE algorithm implemented in the software DNAsp to resolve the gametic phase of highly polimorphic MHC genes. The verification of this methodology is critical for the correct interpretation of the previous chapter.

In chapter 5, we conducted an assessment of the nutritional condition of adult and nestling lesser kestrels in two colonies from the South of Spain.

Glossary

Geolocator: Archival data loggers equipped with an accurate internal clock record light intensities enabling the estimation of sun elevation.

Major Histocompatibility Complex (MHC): A cluster of closely linked genes concerned with antigen production, encompassing two main groups of immune active molecules: class I and II.

Marker: Tool used to answer key questions concerning avian migration (i.e. origin, phenology, route, stopover sites). Two types of markers (intrinsics or extrinsics) can be distinguished depending on the fact if they are naturally inherent to the bird or not.

Migration: The regular, endogenously controlled, seasonal movements between breeding and non-breeding areas. Irregular (nomadism, invasions, irruptions) or unidirectional journeys (juvenil dispersal) are not termed real migration.

Migratory connectivity: Geographic links of individuals or populations between stages of a species's life cycle.

Stable isotopes: Atoms of the same element with different numbers of neutrons, and therefore unique atomic masses.

Stopover: Areas used by migrants to rest, eat or find cover during migration.

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Chapter 1

Geolocators map the wintering grounds of threatened lesser kestrels in Africa

Pre-migratory concentrations of lesser kestrels. Photo: Pepe Antolín

Rodríguez, A., Negro, J.J., Bustamante, J., Fox, J.W. & Afanasyev, V. (2009) Geolocators map the wintering grounds of threatened lesser kestrels in Africa. *Diversity & Distributions* 15: 1010-1016.

Abstract

We used archival light level geolocators (1.5 g) to map the wintering areas and determine some characteristics of the migratory journeys of 20 adult Lesser Kestrels from the Iberian Peninsula. Thirteen geolocators were recovered the following breeding season (2008) after attachment in 2007. Four recovered geolocators provided useful data. According to kernel density analyses, kestrels wintered near the Senegal River (border between Mauritania and Senegal). Pre-nuptial migration took longer than the post-nuptial migration, which may be the consequence of a loop migration. Geolocators have solved a crucial conservation question (i.e., the winter destination of western European Lesser kestrels), and these devices have thus proved useful to determine the location of the winter quarters of small sized migratory species. Our data indicate that European Lesser Kestrels winter in West Africa, in accordance with previous suggestions based on scattered observations during the winter months. This valuable information should serve to focus conservation efforts both in northern Senegal and southern Mauritania. Large roosts gathering thousands of lesser kestrels had been recorded in these areas over the years, but there was no previous confirmation of individuals staying all winter long. Specific and sustained protection of the roost sites, where the birds may be most vulnerable, should be sought in conjunction with local authorities.

Introduction

The Lesser Kestrel *Falco naumanni* is a small migratory falcon which breeds in the Palearctic from the Iberian Peninsula through the Mediterranean basin, Asia Minor, Western Asia, to Mongolia and China. Its populations have decreased dramatically (*c.* 95%) in the western Palearctic since the 1950s, and a reduction of more than 30% of the world population has been estimated, leading to its current Vulnerable status. Habitat degradation and loss, as a result of agriculture intensification, afforestation and urban sprawl in its Western Palearctic breeding grounds, as well as in some winter areas, have been suggested as the main causes of the decline suffered by the species (BirdLife International 2008). However, there is no contrasted information about threat factors in its winter quarters, as the actual location of the wintering grounds in Africa of birds of known origin has never been mapped.

Flocks of foraging Lesser kestrels and roost aggregations have been sighted from November to February in the Sub-Saharan region, East and South of Africa, and South of Arabian Peninsula (Ferguson-Lees & Christie 2001). Moreau (1972) reported that populations from different parts of the breeding range tended to remain separated in the winter; however, evidence for this pattern is sparse and inconclusive. Genetic analyses based on sequencing of mitochondrial DNA indicated that individuals wintering in South Africa originated from the Eastern populations of the breeding range, although the origin of some haplotypes was unknown and several individuals from Western Mediterranean colonies clustered within Eastern populations (Wink *et al.* 2004). The recovery of banded birds in the presumptive African winter quarters seem to support this pattern, but the number of band recoveries to support this hypothesis is low (Appendix S1 A in Supporting Information). Furthermore, migration routes are unknown. Heim de Balsac and Mayaud (1962) hypothesized that Lesser Kestrels from Western European populations carried out a loop migration. Thus, during the post-nuptial migration the individuals would cross the Sahara desert in a wide front, while the return would be mainly carried out through the Western Sahara and/or coastal Africa.

The aim of our study was to locate the wintering areas, as well as the migration routes, of Western European Lesser Kestrels by using light level geolocation (a type of Global Location Sensing - GLS). At present, mapping bird wintering areas of long-distance migratory species is mainly based on banding recoveries or band controls (e.g. Ottosson *et al.* 2005), satellite telemetry if the species is large enough for the individuals to carry PTT's (e.g. Strandberg *et al.* 2008), stable isotope analysis of feathers or other tissues (e.g.

Sarasola *et al.* 2008), and through the use of genetic markers (Wink 2006, Lopes *et al.* 2008). Light level geolocation is a relatively new technology mainly restricted so far to marine animals such as tuna, seals, penguins, albatrosses and shearwaters, which are capable of carrying heavy loggers, are easy to re-capture, and range over large pelagic areas ensuring distance from man-made light sources. Although there is no reason why other animal groups presenting similar characteristics will not be successfully tracked, this technique has not yet been widely used in terrestrial animals (but see Eichhorn *et al.* 2006 and Stutchbury *et al.* 2009). The currently available (and affordable) miniaturized data-loggers make it possible to track and determine migration, stop-over and wintering areas of a wide range of smaller animals with far greater accuracy than is currently possible with other methodologies (see above). Especially for those endangered species which have experienced alarming declines, this information is crucial to their conservation (Newton 2004). Tracking endangered birds to their wintering grounds will help identify threats in these previously unknown areas.

Methods

Light level geolocation is based on logging diurnal changes in light levels (Hill 1994). Archival data loggers equipped with an accurate internal clock record light intensities enabling the estimation of sun elevation. These measurements are used to estimate geographical position (a daily sunrise and sunset recording can give two fixes per day with an average accuracy of 186 ± 114 km - Phillips *et al.* 2004). Day and/or night length determines the latitude and time of local midday and/or midnight the longitude. The loggers measured light every minute, and recorded the maximum light level at the end of every 10 minute period (see details in Afanasyev 2004). The advantages over Platform Transmitter Terminals (PTT) are reduced costs (with no satellite requirements), small size, extended battery life, and if attached securely, indefinite device retention. However, archival light threshold-level geolocation shows several inherent disadvantages: recapture is necessary to download data, and only 2 locations are available per day. In addition, it is impossible to estimate latitude around each equinox, when day time is approximately equal to night time at all latitudes. Furthermore, location accuracy varies according to geographical area, thus latitude determinations are poor between tropics becoming worse closer to the equator, and position cannot be calculated without both a day and night period (Hill 1994).

We used twenty 1.5 g data loggers designed and developed by the British Antarctic Survey (models Mk14S and Mk14 - BAS 2008), which were fitted to 10 Lesser Kestrel pairs during the 2007 breeding season in an urban colony at La Palma del Condado (37º23'N, 6º33'W), Huelva province, southern Spain. Data loggers were attached in two ways: on Teflon harnesses as back mounts (five pairs), and on darvic rings as leg mounts (five remaining pairs). During the 2008 breeding season, we looked for marked birds at the colony to retrieve geolocators and download the data they had accumulated. All retrieved geolocators were pre- and post-calibrated during 7-10 days following manufacturer instructions. Downloading, processing and data analysis were carried out with BasTrak, TransEdit and BirdTracker programs respectively (BAS 2008). Positions were calculated by inspecting the integrity of the daily light curve and marking sunrise and sunset times. Using calibrated data, the sun elevation value for threshold analysis was set to -4.7 degrees corresponding to the arbitrary threshold level to 32. To filter unrealistic positions during the wintering period, the following data points were removed: (a) those obtained from light curves showing interferences at dawn or dusk, and (b) those with a speed index (V_i) above 25 km h^{-1}, as calculated by the square root speed average of the segments formed with the two preceding and the two following positions:

$$V_i = \sqrt{\frac{1}{4}\sum_{j=-2, j\neq 0}^{j=2} (v_{i,j+i})^2}$$

where $V_{i,j+i}$ is the velocity between successive positions *i* and *j+i*.

Data were smoothed twice, and the iterative speed filter then applied to remove the unlikely locations remaining. The great-circle distance between consecutive fixes was used in all velocity calculations (Phillips *et al.* 2004). Kernel density distributions maps were derived from filtered and validated locations using the kernel function implemented in the Animal Movement extension of ArcView 3.2 and a UTM 28N projection. The smoothing parameter (*h*) was set to 45 000 m and grid size to 500 m. Although locations are not serially independent, this is not a requirement for kernel analysis (De Solla *et al.* 1999).

Since Lesser Kestrel migration coincides approximately with the spring and autumn equinoxes, it was not possible to determine migration routes precisely. Therefore, we only took into account the longitude data during the migration time, which are not biased during equinoxes (Hill 1994). In this case, only longitude data from unrealistic positions obtained from light curves showing interferences at dawn or dusk were deleted. Timing and rate of migration were calculated assuming birds finished migration when longitude stabilized (Guilford *et al.* 2009, Stutchbury *et al.* 2009). This assumption is certainly not valid, but lets us compares the patterns of post- and pre-nuptial migrations.

Results

At least 15 different individuals carrying geolocators were sighted during the 2008 breeding season at the colony. We were able to retrieve 13 geolocators, of which six failed to download or only contained data of a few days after attachment, largely due to physical damage. Out of seven birds recaptured with geolocators which contained some data, one did not migrate and remained in the Iberian Peninsula (see Appendix S2). Geolocators fitted as leg mounts on darvic rings (n = 7) showed damage caused by bites, scratches and ingrained dirt, and none contained usable data. However, recorded data in three of these damaged leg mounted geolocators suggested that the birds did migrate, and that they probably wintered in the same general area as the other birds with back-mounted geolocators.

The detailed migration results discussed here came from three harness mounted birds. All individuals (n = 3) wintered in the same area in the North of Senegal and South of Mauritania (Fig. 1). They were present in the area from the end of September until early March. Home range individually varied in area, but there was a partial overlap in the winter areas of the three individuals (Appendix S3). These figures are probably exaggerated due to unquantifiable shading uncertainties (e.g. vegetation, clouds, dirt) adding to the inherently low accuracy of geolocators.

According to longitude data, the post-nuptial migration took place during the second half of September and early October, and lasted approximately 5 ± 1 days (n = 3). The pre-nuptial migration took place during the first half of February and late March (Fig. 2), and lasted approximately 24.3 ± 10 days (n = 3) (Appendix S2). The length of pre-nuptial migration was 4.2 times longer and 3.5 times more variable in time than the post-nuptial one.

Discussion

Despite the constraints inherent to light level geolocation, this study shows for the first time the wintering areas of Lesser Kestrels with a known origin (i.e., a colony in the south of the Iberian peninsula), as well as the first details of timing and rate of their migrations. So far, Lesser Kestrels had been widely recorded in West Africa, but not consistently (Moreau 1972, Pilard *et al.* 2004, 2005). Specifically in Senegal, large flocks of Lesser Kestrels had

been sighted in the deltas of the Senegal (Triplet *et al.* 1993, Triplet & Yésou 1995) and Saloum rivers (Isenmann 2005, LPO 2008; see Appendix S1 B). These observations had been carried out during January or February, and it was believed that the birds were in active migration (Pilard *et al.* 2004). Our data show that Lesser Kestrels may spend the whole winter in those areas. Possibly, the observed large flocks reflect pre-migratory aggregations, as well as the use of communal roosts at times when migratory locusts are the staple prey (Triplet *et al.* 1993, Triplet & Yésou 1995, Isenmann 2005). Ringing of Lesser Kestrels has provided only five recoveries in the presumptive winter quarters (Appendix S1 A), two corresponding to western European birds (see below) and three to Asian birds that wintered in South Africa. In the case of Spain, more than 37,000 Lesser Kestrels have been ringed during the period 1973-2006, and only two recoveries of corpses in unusual dates (20 June 1992 and 23 April 1996) have been obtained in the Western African presumptive winter quarters. The scarcity of recoveries may be associated to the low presence of ornithologists, birdwatchers or even tourists in the Sahel area, at least compared to other African regions.

The proportion of individuals which migrated was similar (75% or 85% if we take into account the darvic ring data-loggers with poor data) to that reported for the species in the same population (19% of adults are residents in southern Spain; Negro *et al.* 1991, Negro 1997). The fact that Lesser Kestrel migration coincided approximately with the equinoxes, and in proximity to the tropics, precluded the ability to include latitude in the plotting of migratory routes (Hill 1994). The longer pre-nuptial migration in comparison to the post-nuptial migration contrasts with the typical pattern shown by other birds (Curry-Lindahl 1981, Alerstam *et al.* 2006, Stutchbury *et al.* 2009). In migratory birds, early arrivals on the breeding grounds entail advantages in terms of high-quality site occupancy. Several facts, including the active defence of nest holes during a three month period before egg-laying (February, March and April) or the sequential arrival of adult males, adult females and yearlings to the breeding colonies (Negro *et al.* 1991, Negro 1997), would predict a migratory pattern contrary to the observed one (see also Sergio *et al.* 2007). Northern-east directions of dominant trade winds through the migratory routes could be responsible for the observed pattern with tail-winds aiding the post-nuptial migration (Liechti 2006). Another plausible and non-mutually exclusive explanation for our results is the ringlet migration proposed by Heim de Balsac and Mayaud (1962). Thus, the rapid rate of change in longitude during post-nuptial migration could indicate that the birds migrate in a straight southerly direction crossing the Sahara desert, and ending the migration in a relatively short travel through the Sahel until the arrival to the winter areas. On the other

hand, during the pre-nuptial migration, Lesser Kestrels may flock together and come back to the breeding grounds through Western Sahara, and thus a gentler longitude slope would be drawn (Fig. 2). The mean velocity of post-nuptial migrations reported here (range 4-6 days and 417-625 km/day) are higher than the estimated for other *Falco* species (McGrady *et al.* 2002, Ganusevich *et al.* 2004, Strandberg *et al.* 2009b) or raptors in general (maximum speed of about 200 km, see Strandberg *et al.* 2009a) and similar to two songbirds (Stutchbury *et al.* 2009). This may be related to the fact that the Sahara desert constitutes almost entirely the route until the wintering grounds. It is well known that birds cross the Sahara desert in a shorter time period than other safer and more suitable zones (Meyburg *et al.* 2004, Klaassen *et al.* 2008). In the case of Lesser Kestrel, it has been proposed that birds make a continuous flight of some 2500 km in post-nuptial migration (Moreau 1972). In this sense, Eurasian Hobby *Falco subbuteo* is able to make a nonstop flight over a distance of 740 km across the Mediterranean Sea during 27 hours (Strandberg *et al.* 2009b). If we accept the loop migration hypothesis, the pre-nuptial migratory route will cross a smaller area of desert, and consequently birds could fly over a safer terrain. However, we have only used longitude data, and therefore, our conclusions may be biased.

The fitted geolocators did not appear to have severely affected the birds in any significant way. Breeding success during tagging year and survival did not vary between tagged and un-tagged individuals (Rodríguez *et al.* unpublished data). We will therefore assume that data obtained are representative for breeding birds of our study colony. Given that the geolocators fitted on darvic bands failed to provide usable data due to damage caused by the birds themselves, we recommend the use of back mounted geolocators, at least for raptors or species with strong beaks. Furthermore, leg mounting may be unsuitable for geolocators on many terrestrial species due to the accumulation of dirt over the light sensor; to date, most success with leg mounting geolocators has been with seabirds (e.g. Guilford *et al.* 2009). In the case of back mounted geolocators, the light sensor is kept cleaner and less accessible to the beak and talons than in the leg mounts.

According to mostly anecdotal observations, the winter ecology of the Lesser Kestrel appears to be similar to that of other resident or long distance migratory raptors such as the Black Kite *Milvus migrans* or the African Swallow-tailed Kite *Chelictinia riocourii* in the same area, roosting in large communal roosts and feeding on locusts and grasshoppers (Triplet & Yésou 1995, Pilard *et al.* 2004, 2005, Isenmann 2005, LPO 2008). It has been reported by satellite telemetry that other Western European raptors such as the Marsh Harrier *Circus aeruginosus* (Strandberg *et al.* 2008), Montagu's Harrier *Circus pygargus* (Limiñana *et al.* 2007) or Egyptian Vulture *Neophron percnopterus* (Meyburg *et al.* 2004),

winter also in the Sahel zone. Some of them, mainly locust and grasshopper consumers, have suffered severe declines in recent decades, possibly related to droughts and pesticide use to control insects (Newton 2004, Sánchez-Zapata *et al.* 2007). Due to the gregarious behavior of kestrels, specific and localized conservation measures may help conserve almost the entire wintering European population (LPO 2008).

While the banding of thousands of Lesser Kestrels throughout the Western European breeding range for more than 30 years has failed to provide conclusive data on wintering and migration, inexpensive geolocators have solved a crucial question in only one year of study. Because migratory kestrels spend a considerable time on the wintering grounds, this valuable information should serve to focus conservation efforts both in time and space. Specifically, the large aggregations of kestrels previously observed in northern Senegal and southern Mauritania in the winter months may now be attributed to genuine wintering individuals that may stay in the area for several months. A single roost site found in Senegal gathering about 24,000 individuals in 2007, and that may have been used by the kestrels for years, holds every season more than the equivalent to one third of the western European lesser kestrel population and deserves specific protection in conjunction with local authorities (see LPO 2008).

It is now clear that there are at least two main wintering grounds for lesser kestrels in Africa: the one reported here in Western Africa, that appears to recruit birds from the Western Palearctic; and South Africa, the first destination for Lesser Kestrel recognized years ago, and that seems to hold birds of Asian origin exclusively (Wink *et al.* 2004). The western route is considerably shorter (2,500-3,500 km) than the eastern one (8,000-10,000 km), raising interesting questions on energetic constraints and adaptations for medium or long-distance migration in birds travelling one or the other migration route.

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Figures

Fig. 1 Validated locations and activity ranges derived from kernel analyses (encompassing 95%, 75% and 50% of the locations) in the wintering areas of three adult Lesser Kestrels during a winter period (November, December 2007 and January 2008). The white circle with a black point shows the location of the breeding colony of the individuals at La Palma del Condado (Huelva Province, Spain).

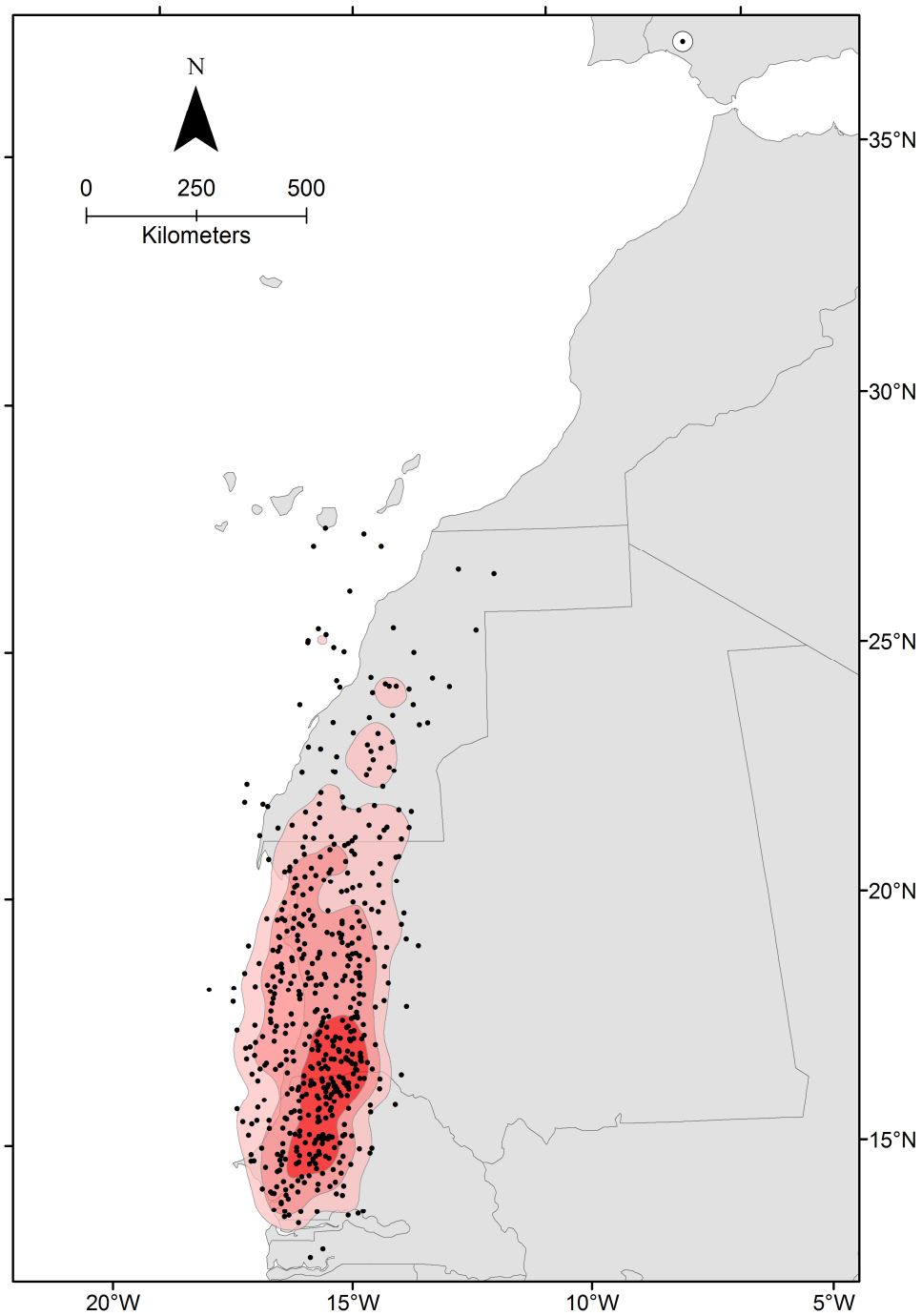
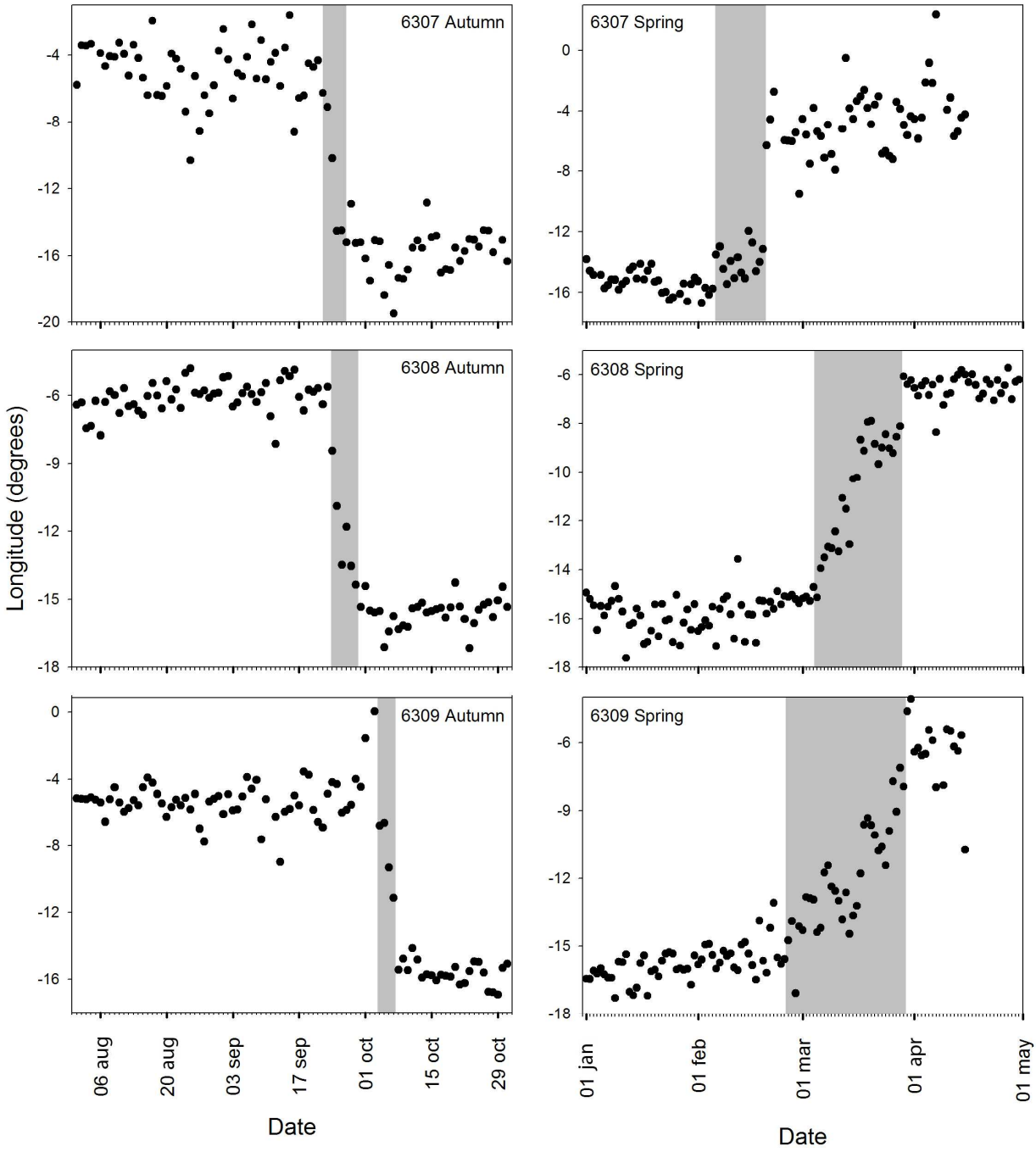
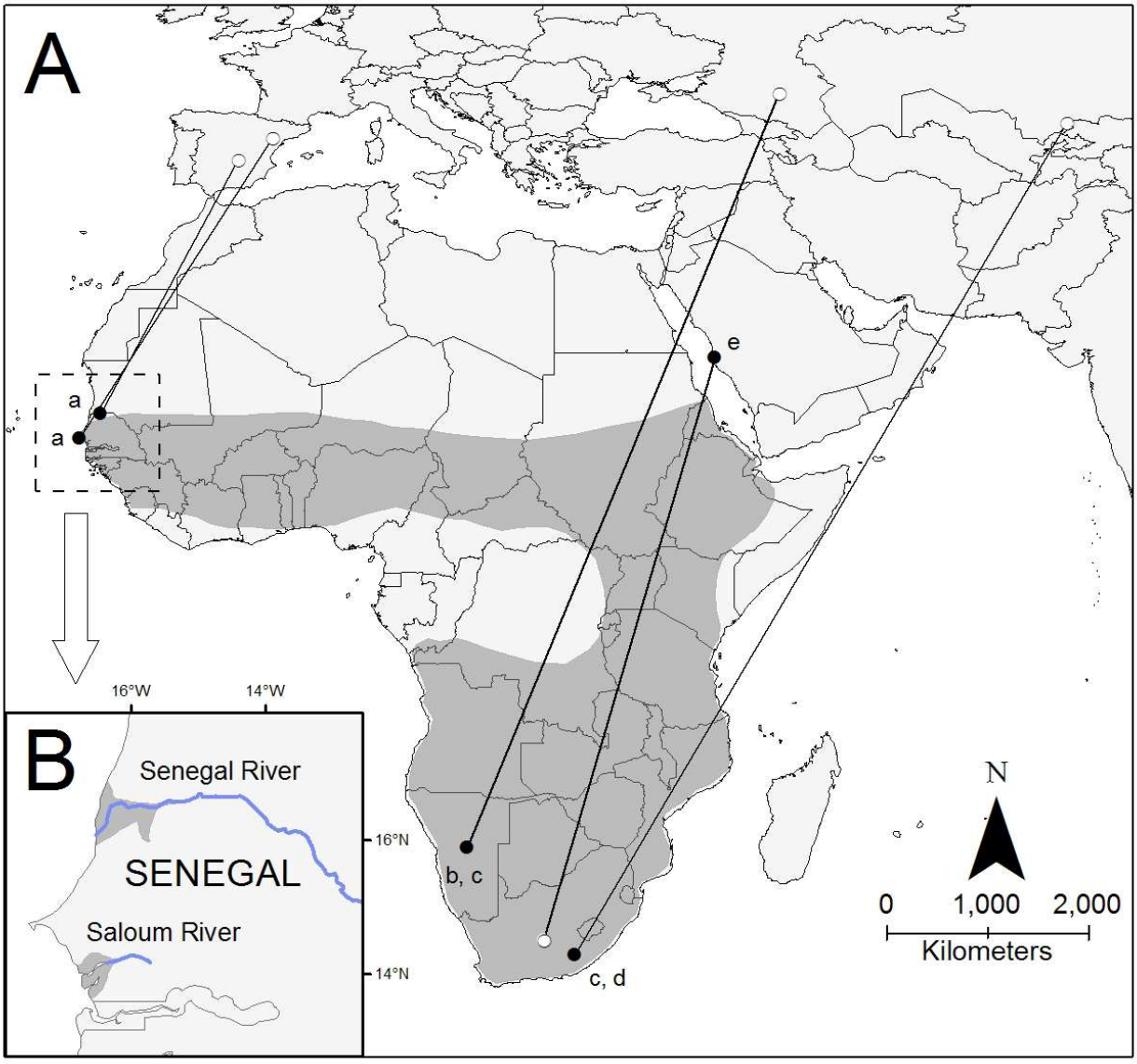


Fig. 2 Longitude variations during post and pre-nuptial migration of Lesser Kestrels. Shade area indicates the estimated duration of migration. Each point corresponds to the average longitude of the date (two locations per day).



Supporting Information

Appendix S2 (A) Band recoveries of migratory Lesser Kestrels *Falco naumanni* in wintering areas. Data from the Migratory Species Office[a] (Spanish Ministry of Environment), EURING data bank[b], SAFRING[c] (South African Bird Ringing Unit), Preston[d] (1976) and Anonymous[e] (1997). Grey area shows putative wintering areas of the Lesser Kestrel according to Ferguson-Lees and Christie (2001). (B) Locations of Senegal and Saloum rivers (blue) and deltas (grey).



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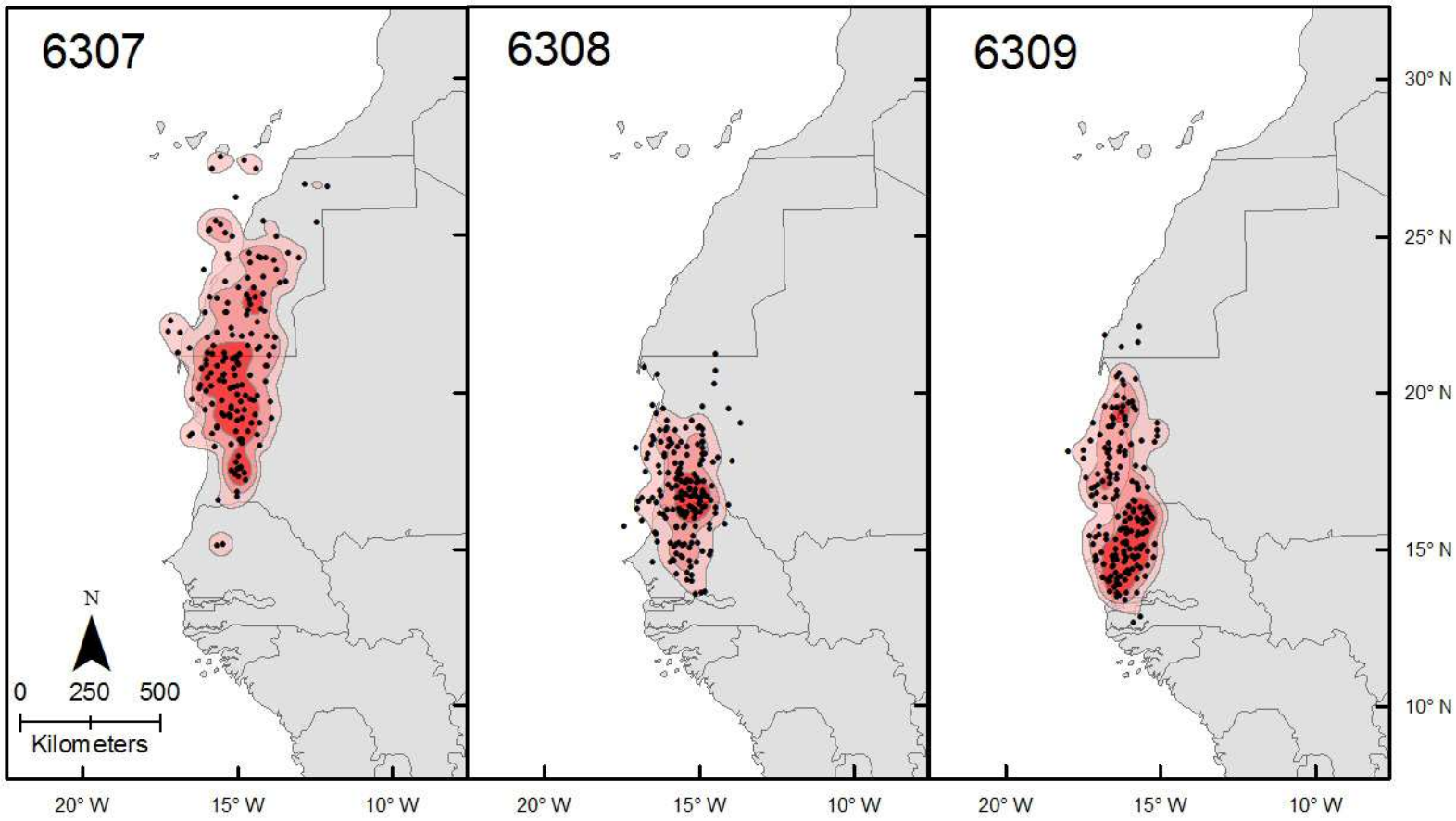
Appendix S2 Distances and duration of pre- and post-nuptial migration of Lesser Kestrels. Type, state at recovery and ID of the geolocators is included, as well as sex of the bearer.

ID	Type	Sex	State	Mode	Data	Migration	Post-nuptial migration duration (days)	Pre-nuptial migration duration (days)	Post-nuptial migration speed (km/day)	Pre-nuptial migration speed (km/day)
6302	harness	F	Damaged	Asleep	-	-	-	-	-	-
6306	harness	M	ok	Collecting data	Good	No	-	-	-	-
6307	harness	M	ok	Collecting data	Good	Yes	5	14	500	179
6308	harness	M	ok	Collecting data	Good	Yes	6	25	417	100
6309	harness	F	ok	Collecting data	Good	Yes	4	34	625	74
6310	harness	M	Damaged	Asleep	-	-	-	-	-	-
6349	ring	M	Dirty	Asleep	-	-	-	-	-	-
6351	ring	M	ok	Asleep	-	-	-	-	-	-
6353	ring	M	Scratched and dirty	Collecting data	Bad	Yes	-	-	-	-
6355	ring	F	Scratched and dirty	Collecting data	Bad	Yes	-	-	-	-
6356	ring	F	Damaged and dirty	Asleep	-	-	-	-	-	-
6357	ring	F	Damaged and dirty	Asleep	-	-	-	-	-	-
6358	ring	F	Scratched and dirty	Collecting data	Bad	Yes	-	-	-	-

Appendix S3 Validated locations and activity ranges derived from kernel analyses (encompassing 95%, 75% and 50% of the locations) for each individual in the winter areas of three Lesser Kestrels during a winter period (November 2007 to January 2008).



Chapter 2

Effects of geolocator attachments on breeding parameters of lesser kestrels *Falco naumanni*

An adult male lesser kestrel incubating at Manzanilla colony. Photo: Airam Rodríguez

Rodríguez, A., Negro, J.J., Fox, J.W. & Afanasyev, V. (2009) Effects of geolocator attachments on breeding parameters of Lesser Kestrels *Falco naumanni*. *Journal of Field Ornithology* 80: 399-407.

Abstract

Light level geolocators, also known as GLS loggers, are electronic devices intended for tracking the location of wide-ranging animals using ambient light to estimate latitude and longitude. Miniaturized geolocators have become available recently that may be used on relatively small migratory birds. However, information on potential harmful effects of geolocators on their bearers is scarce. The effect of 1.5 g geolocators (dimensions: 21 × 6.5 × 9 mm) on breeding rates, nutritional condition of nestlings and survival of migratory Lesser Kestrels *Falco naumanni* was assessed during an annual cycle. Geolocators were fitted in spring 2007, during the breeding season, using two attachment methods (Teflon wing harnesses and darvic rings), and they were removed in 2008 as soon as the birds were located and captured after the pre-nuptial migration. No differences in breeding rates of control and tagged pairs were detected during the 2007 breeding season, but tagged pairs showed an increase of fledgling mortality in the following nesting season. Furthermore, nestlings of tagged individuals showed higher triglycerides and uric acid concentrations in blood than control nestlings during the breeding season following tagging. As for return rates, 75% of tagged birds came back to the colony after the non-breeding period a proportion that does not differ from previous estimates for the species. Although back-mounts are slightly heavier and require more skill to fit them, we propose their use on small migratory raptors or other similar sized and terrestrial birds, given that most of our leg-mounted geolocators were heavily damaged and dirty when recovered, failing to provide usable data.

Introduction

Telemetry studies can provide valuable information about the behavior and ecology of birds, but the effect of devices used to track animals is often assumed to be negligible (Murray and Fuller 2000). Studies where tag impact has been considered have revealed variation among species, with some reporting no adverse effects (Hiraldo *et al.* 1994, Terhune *et al.* 2007, Anich *et al.* 2009) and others demonstrating effects on breeding behavior, predation rates, breeding success, survival, and hunting skills (e.g., Whidden *et al.* 2007). Among raptors, tags have been found to have negative effects on the survival of Northern Goshawks (*Accipiter gentilis*) and Prairie Falcons (*Falco mexicanus*; Reynolds *et al.* 2004, Steenhof *et al.* 2006), as well as on the types of prey delivered to nests by Prairie Falcons (Vekasy *et al.* 1996).

Light-level geolocation technology has recently been used on terrestrial birds to investigate long-distance movements (Eichhorn *et al.* 2006, Stutchbury *et al.* 2009, Rodríguez *et al.* in press). To determine if data derived from the use of geolocators are unbiased due to possible handicaps on the carriers and to ensure the well-being of the birds being studied, the possible effects of geolocator tags on birds need to be examined.

We examined the possible effects of geolocators on various breeding parameters (clutch size, number of fledged young, and clutch initiation date) of Lesser Kestrels (*Falco naumanni*) during two consecutive nesting seasons. Because the geolocators used represented less than 3% of Lesser Kestrels' body mass (less than the generally accepted 5% threshold; Kenward 2001), we predicted that breeding parameters would not be affected. We also evaluated selected blood biochemistry parameters (triglycerides, cholesterol, urea and uric acid) of nestlings of tagged and control pairs because differences in these parameters may indirectly indicate subtle effects of geolocators on adult breeding behavior. Finally, because the effect of devices can be influenced by where or how they are attached (Murray and Fuller 2000), two attachment methods for the GLS loggers were compared: Teflon wing harnesses and darvic plastic leg bands.

Methods

Geolocation and model species

Light-level geolocation uses ambient light to estimate latitude and longitude, determined by day and night lengths and time of local midday or midnight, respectively. Light-level

geolocators are equipped with an accurate internal clock that is used to time-stamp measurements from a photoreceptor.

Lesser Kestrels are small, partially migratory falcons that breed colonially in holes and crevices of buildings in western Europe. During February and March, birds arrive at breeding colonies from their wintering grounds. Egg laying typically occurs between late April and early May. After 28-32 days of incubation, hatching occurs during June and young fledge during the first half of July. Both males and females share incubation and brooding duties. These kestrels are sexual dimorphic and dichromatic, with males more brightly colored and lighter than females (ranges = 90-172 g and 138-208 g for males and females, respectively). Populations have decreased dramatically (about 95%) in the western Palearctic since the 1950s, and a reduction of more than 30% of the world population has been estimated, explaining its current Vulnerable status (Negro 1997).

Experimental procederes

We studied a colony (about 25-30 pairs) located on a cereal silo within the urban area of La Palma del Condado (37º23'N, 6º33'W), Huelva province, in southern Spain. Nests were located on the window ledges of the building, allowing us to capture kestrels by hand at their nests and to accurately assess breeding parameters.

During June 2007, 20 adult Lesser Kestrels representing 10 randomly chosen breeding pairs were fitted with geolocators designed and created by engineers from the British Antarctic Survey (www.birdtracker.co.uk). Weight and dimensions of the geolocators were 1.5 g and 21 × 6.5 × 9 mm, respectively, excluding the sensor stalk. Five randomly selected pairs were fitted with Mk14S (light sensor on stalk) devices on harness attachments, and five pairs were fitted with Mk14 (no stalk) devices on darvic plastic rings on the legs (see below). The remaining 14 pairs breeding in the silo colony in 2007 were used as controls. Most adult kestrels were captured when brooding 1-7 day old chicks (from 1 - 22 June 2007) at their respective nest sites.

We used two different methods to attach loggers to kestrels: Teflon wing harnesses and darvic plastic leg rings. The former were constructed with cotton thread, cyanoacrylate glue, and approximately 30 cm of 4.75-mm-wide tubular Teflon ribbon (Biotrack Ltd., Dorset, UK) and doing a frontal knot in the bird breast (M. de la Riva, Estación Biológica de Doñana CSIC, pers. comm., Kenward 2001; Fig. 1A, B). The mean weight of the harness plus geolocator was 3.09 ± 0.03 (SD) g. For the second method, geolocators were attached with a weatherproof cable tie (TY523MXR; Thomas & Betts, Memphis, TN) to a darvic ring on the

bird's leg (Fig. 1C). Darvic rings were provided by the Ringing Office of Doñana Biological Station, and their size and weight were 17.5 × 10 mm and 0.9 g. The mean weight of these attachments was 2.44 ± 0.08 (SD) g.

During the 2007 and 2008 breeding seasons, we monitored the colony to record clutch initiation dates, clutch sizes, and the number of fledged young per active pair. From February-April 2008 (before egg laying), we used a spotting scope (×30) and binoculars (×10) to search for tagged birds and locate nest-sites (i.e., window ledges). Birds were captured at night and most tags were removed between 1 March and 15 April period (before egg-laying). At the time of capture, selected body measurements and mass were recorded.

During the 2007 and 2008 nesting seasons, a blood sample (0.5 ml) was taken from each nestling and immediately refrigerated. To minimize possible effects of circadian rhythms on parameter levels, all blood samples were collected between 08:00 and 14:00 hours. Within 6 hrs of sampling, blood samples were centrifuged for 10 min at 4500 g, and the plasma was separated and stored at -20ºC. Plasma was analyzed for triglycerides, cholesterol, urea, and uric acid using a Screen Point autoanaliser (Hospitex Diagnostics, Sesto Fiorentino, Italy), and commercial kits from Biolabo labs (Maizy, France). Plasma biochemical analyses were performed by Wildvets S.L.P. (Seville, Spain).

Statistical analices

We used two-way ANOVA to test for differences in the geolocator/body mass ratio (i.e., harness/darvic included) during tagging (with sex and geolocator type as factors), and in the body mass of tagged versus untagged individuals (with sex as a factor). Because variables were not normally distributed, possible differences in clutch date, clutch size and productivity (number of fledged young) among groups were examined separately using Mann-Whitney *U*-tests. To avoid possible differences in productivity due to clutch size, we also assessed the productivity/clutch size ratio using Mann-Whitney *U*-tests. Given our small sample sizes, we calculated the statistical power (*w*, probability of obtaining a significant result when the hypothesis is false) following the methodology employed by Jennions and Møller (2003), as well as the difference between effect size of our data (ES) and effect size required to be detected with high power (0.80) given our sample sizes (ES_{min}). Tests for possible differences in the breeding parameters (clutch date, clutch size, fledged young, and productivity/clutch size ratio) of tagged and untagged birds were one-tailed because the geolocators effect was expected to be negative. For comparison of the

possible effects of attachment method (harnesses and rings) on breeding parameters, tests were two-tailed because no directional change was expected. We used Linear Mixed Models to test the possible effects of parental status (geolocators vs. controls) on the body condition (weight and plasma biochemical parameters) of nestlings. Age and the number of siblings were included as covariates, and nest identity as a random factor to avoid pseudoreplication. Age (in days) was estimated using the eighth primary (mm) according to the function AGE = 10.44 + 0.14*EIGHTH PRIMARY (Negro 1997). Biochemical variables were Log transformed when assumptions of parametric statistics (normality and homocedasticity) were not met. Adult recapture asynchrony during 2008 precluded a comparison of plasma biochemical parameters of tagged versus untagged adults. In 2008, pairs that included at least one kestrel that was tagged in 2007 were compared to the remaining pairs in the colony.

Results

Because of the sexual size dimorphism, the attachments represented a greater burden for male Lesser Kestrels than females ($F_{1, 16}$ = 61.8, P < 0.001), and harness attachments were heavier than those on darvic rings ($F_{1, 16}$ = 154.1, P < 0.001) during tagging in 2007. No interaction between factors was detected ($F_{1, 16}$ = 2.0, P = 0.18; Fig. 2). Small sample size of returning birds precluded assessment of these differences in 2008, but the pattern was similar to that in 2007 (Fig. 2).

Fifteen of 20 birds (75%) tagged in 2007 were re-sighted in the colony during the 2008 breeding season, with 14 of those 15 re-captured and 13 geolocators recovered (see below). Despite differences in geolocator/body mass ratios, return rates of the birds did not differ with either attachment type (seven harnesses and eight darvic rings) or sex (eight males and seven females). We found no difference in the body mass of tagged and untagged individuals during the 2008 pre-laying period ($F_{1, 35}$ = 0.08, P = 0.79; Fig. 3).

Only one kestrel had an injury at the time of recovery. This bird, a female, had a small wound on the breast, probably due to a bad harness fit. When we removed her harness, the frontal knot was partly embedded in the underlying tissue (Fig. 1D). In addition, one male fitted with a leg-mounted geolocator was found apparently exhausted in late summer, two months after being banded, and well after his brood of five nestlings had fledged. This male was found 140 km north of the colony by a private citizen, and admitted to a wildlife rehabilitation center. After about six months, it was released on 2 February 2008 with the

geolocator removed. This individual returned to the silo colony and successfully fledged three young in 2008.

At least 10 of the returned and tagged kestrels bred successfully in 2008, rearing at least one fledgling (this time without the geolocators because they were all removed). The remaining five individuals were captured or sighted in the colony before the egg-laying period (February-April), but we were not able to determine if they bred. In 2008, most previously tagged birds (N = 8) paired with a different mate. However, one pair of kestrels remained together and nested in the same cavity as in 2007.

We found no significant differences between pairs with attachments and controls in clutch size and number of fledged young (Table 1). In 2008, the productivity/clutch size ratio varied (Table 1), but clutch initiation date did not differ between experimental groups (U = 33.0, P = 0.29, w = 18.7 %, ES-ES_{min} = -0.87). As expected (because birds to be tagged were captured after hatching and pairs were randomly selected), we detected no differences in clutch sizes between pairs with tags and control pairs during the 2007 breeding season. However, marginal significant differences were detected in clutch size and the number of young fledged for pairs with different attachment methods (Table 2). Pairs with harness attachments had a lower breeding success, although the productivity/clutch size ratio was similar (Table 2). During the 2008 nesting season, nestlings of tagged pairs had higher concentrations of triglycerides and uric acid than nestlings of untagged birds, but the body mass of nestlings was similar between experimental groups (Table 3).

When recovered, some geolocators were damaged. All leg-mounted geolocators (N = 7) had scratches, peck marks, and dirt, and three had been destroyed. However, geolocators mounted on harnesses had no scratches or dirt (N = 6), and only one had been damaged (missing light sensor).

Discussion

Light-level geolocator tags representing 1.4-2.7% of body mass did not affect the breeding success of adult Lesser Kestrels in our study during the year they were tagged. Similarly, previous studies have revealed no effects of 3-5 g tail-mounted radio-tags on breeding Lesser Kestrels (Hiraldo *et al.* 1994), and of back-mounted radio-tags on other falcons (Vekasy *et al.* 1996) during the year of marking, as well as the general recommendation establishing that device load should not exceed 4-5% of body mass (Kenward 2001). However, pairs of Lesser Kestrels with at least one tagged member fledged fewer young in

the following breeding season. Such results are difficult to explain because we found no differences in body mass between tagged and untagged birds during the 2008 pre-laying period, but the difference in fledging rates may be related to annual environmental conditions. Mean breeding success for the entire colony (fledged young per breeding attempt) was higher in 2007 (3.21 ± 1.25, N = 25) than in 2008 (2.84 ± 1.66, N = 31), as was the mean body mass of fledglings. Thus, when conditions were favorable, no effects of geolocators were detected (2007), but, with less favorable conditions, their effects may have been more apparent (Murray and Fuller 2000).

The productivity/clutch size ratio did not vary for pairs of kestrels with different attachment methods (i.e., harnesses and darvic rings), suggesting that the marginal differences in clutch size and productivity may have been due to the small sample size (note that clutch size was recorded before tagging kestrels; see Table 2). In addition, the high return rates in 2008 suggest that geolocator attachment had little or no effect on kestrel flight capacity. In fact, return rates in our study were similar to those reported in a previous and larger study of the same population (Hiraldo *et al.* 1996; see also Negro 1997). Given that breeding success and return rates were not affected by attachment type (harnesses or darvic plastic rings), we recommend the use of back-mounts (at least for raptors or other species with strong bills), even though they are slightly heavier and attaching them requires more skill than leg-mounted geolocators. Back-mounted geolocators have provided crucial information concerning the wintering areas of this threatened kestrel. However, most leg-mounted geolocators in our study were heavily damaged and dirty when recovered and did not provide usable data (Rodríguez *et al.* in press).

The apparent similarity in nestling condition (nestling body mass was similar between experimental groups) suggests that the higher concentrations of triglycerides and uric acid in nestlings of tagged pairs might be due to differences in types of prey delivered to the nest, possibley a consequence of a post-tagging effect of geolocators on the behavior of parents. Prey delivery rates of radio-tagged and untagged adults were similar in other studies involving this and other species (Hiraldo *et al.* 1994, Vekasy *et al.* 1996), but the types of prey brought to the nest differed between the two experimental groups (Vekasy *et al.* 1996). Another possible explanation, not mutually exclusive, is that tagged birds incurred a delayed handicap during the 2008 mating season and, as a result, might have been more likely to mate with poor quality individuals. In support of this conclusion, we found that differences were not significant in 2007 when pairing occurred before attachment of the geolocators.

To date, the use of light level geolocators to investigate the migratory strategies of birds has largely been limited to relatively large species (Croxall *et al.* 2005, Eichhorn *et al.* 2006, Shaffer *et al.* 2006, González-Solís *et al.* 2007). As their weight and size decreases, geolocators can be used on smaller species. However, their possible negative effects must be tested, especially with small species with greater attachment to bird mass ratios. To our knowledge, Lesser Kestrels are one of the lightest species in which these loggers have been used so far and their effects evaluated (see Igual *et al.* 2005 and Rayner 2007 for larger species, and Stutchbury *et al.* 2009 for smaller ones). Despite of effects caused by geolocators during the following breeding season, we think that the provided information by them in our study justifies its use, due to the lack of knowledge concerning migration and winter ecology (Rodríguez *et al.* in press). It should be noted that the species has a disappointingly low recovery rate of banded birds. Out of 37,000 Lesser Kestrels banded in Spain during the period 1973-2006, only two recoveries have been made in the presumptive African winter quarters (Oficina de Especies Migratorias, pers. comm.). Lesser Kestrels are particularly well suited for using geolocation because this technique relies on the ease of recapturing of tagged birds after a protracted period of time. As with many seabirds, where use of geolocators is more common, adult Lesser Kestrels are extremely philopatric (Negro *et al.* 1997, Serrano *et al.* 2001) and tend to return to breed at the same colony where they bred the previous year. Other small raptors whose migration might be tracked using geolocation are the colonial Red-legged Falcons (*Falco vespertinus*) and Amur Falcons (*F. amurensis*), as well as small migratory owls, such as Scops Owls (*Otus scops*). However, loggers must be retrieved and downloaded, and therefore, the probability of recovery of fitted birds must be taken into account in the design of studies. In addition, researchers should evaluate the trade off between possible harmful effects on their model species and potential information that they might obtain (Murray and Fuller 2000).

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Tables

Table 1 Breeding parameters of geolocator-tagged and control Lesser Kestrel *Falco naumanni* pairs at the Silo colony (Huelva province, southern Spain). Geolocator were deployed during the 2007 hatchling and 2008 before egg-laying period. In 2007, clutches were recorded before tagging kestrels, while in 2008, breeding parameters were recorded after deployment (see 'Methods'). Statistical and *P*-values are shown.

	Controls			Geolocator			*U*-test	*P*-Value	Power %	Es-Es_{min}
	Mean	SD	*N*	Mean	SD	*N*				
2007										
Clutch size	4.36	0.76	14	4.20	1.03	10	67.5	0.34	10.9	-0.886
Fledged young	2.93	1.39	14	3.60	0.97	10	58.5	0.18	36.8	-0.504
Productivity/clutch size ratio	0.69	0.32	14	0.86	0.15	10	54.0	0.13	47.9	-0.383
2008										
Clutch size	4.00	0.82	10	4.50	0.76	8	26.0	0.12	35.6	-0.601
Fledged young	3.70	1.06	10	3.25	1.04	8	33.0	0.29	21.8	-0.804
Productivity/clutch size ratio	0.92	0.14	10	0.70	0.16	8	16.0	0.017	90.4	0.23

Table 2 Breeding parameters of Lesser Kestrel pairs tagged with either harnesses or darvic rings at the Silo colony (Huelva province, southern Spain) in 2007. Clutch sizes were recorded before tagging kestrels (see 'Methods').

	Harnesses			Darvic rings			*U*-test	*P*-values	Power %	ES-Es_{min}
	Mean	SD	*N*	Mean	SD	*N*				
Clutch size	3.60	0.89	5	4.80	0.84	5	4.0	0.095	48.7	-0.637
Fledged young	3.00	0.71	5	4.20	0.84	5	3.5	0.055	57.3	-0.481
Productivity/clutch size ratio	0.84	0.15	5	0.88	0.16	5	11.0	0.84	6.5	-1.766

Table 3 Summary statistics and linear mixed model results (using 'age' and 'number of siblings' as fixed factors and 'nest identity' as random factor) of plasma biochemical parameters and mass of nestling Lesser Kestrels from control *versus* geolocator-attached pairs.

Biochemistry parameters	Control			Geolocator			*F*	*P*	Confidence intervals
	Mean	SD	*N*	Mean	SD	*N*			
2007									
Mass (g)	151.3	19.0	42	149.9	13.4	36	1.97	0.18	[-4.02 , 20.25]
Triglycerides[a] (mg dl^{-1})	341.4	210.2	39	335.1	229.5	36	0.24	0.63	[-0.13 , 0.21]
Cholesterol[a] (mg dl^{-1})	190.6	67.4	37	191.5	48.4	36	0.02	0.90	[-0.10 , 0.11]
Urea (mg dl^{-1})	14.3	3.5	36	12.6	4.2	36	2.97	0.10	[-0.43 , 4.16]
Uric acid (mg dl^{-1})	16.4	5.6	35	15.4	6.0	35	0.46	0.51	[-2.85 , 5.58]
2008									
Mass (g)	145.2	20.3	34	144.3	15.2	27	0.02	0.90	[-15.3 , 13.6]
Triglycerides[a] (mg dl^{-1})	226.5	168.7	29	292.7	120.1	26	11.3	0.005	[-0.39 , -0.08]
Cholesterol[a] (mg dl^{-1})	200.2	44.9	29	226.5	47.2	26	2.89	0.12	[-0.12 , 0.01]
Urea (mg dl^{-1})	15.8	7.0	29	21.7	9.7	26	4.25	0.06	[-12.1 , 0.31]
Uric acid (mg dl^{-1})	16.7	5.2	29	19.8	5.5	25	7.80	0.018	[-8.28 , -0.96]

[a]Variable Log transformed.

Figures

Fig. 1 Adult female Lesser Kestrel *Falco naumanni* with a Mk14S geolocator attached in a Teflon harness (2007, fitting day). B, Adult male Lesser Kestrel with a geolocator attached in a Teflon harness (2008, at recovery after wintering and migration). C, Adult female Lesser Kestrel with a Mk14 geolocator attached in a darvic ring (2007, fitting day). The GLS unit dimensions are 21 × 6.5 × 9 mm excluding the sensor stalk. D, Small wound in the breast of a female kestrel.

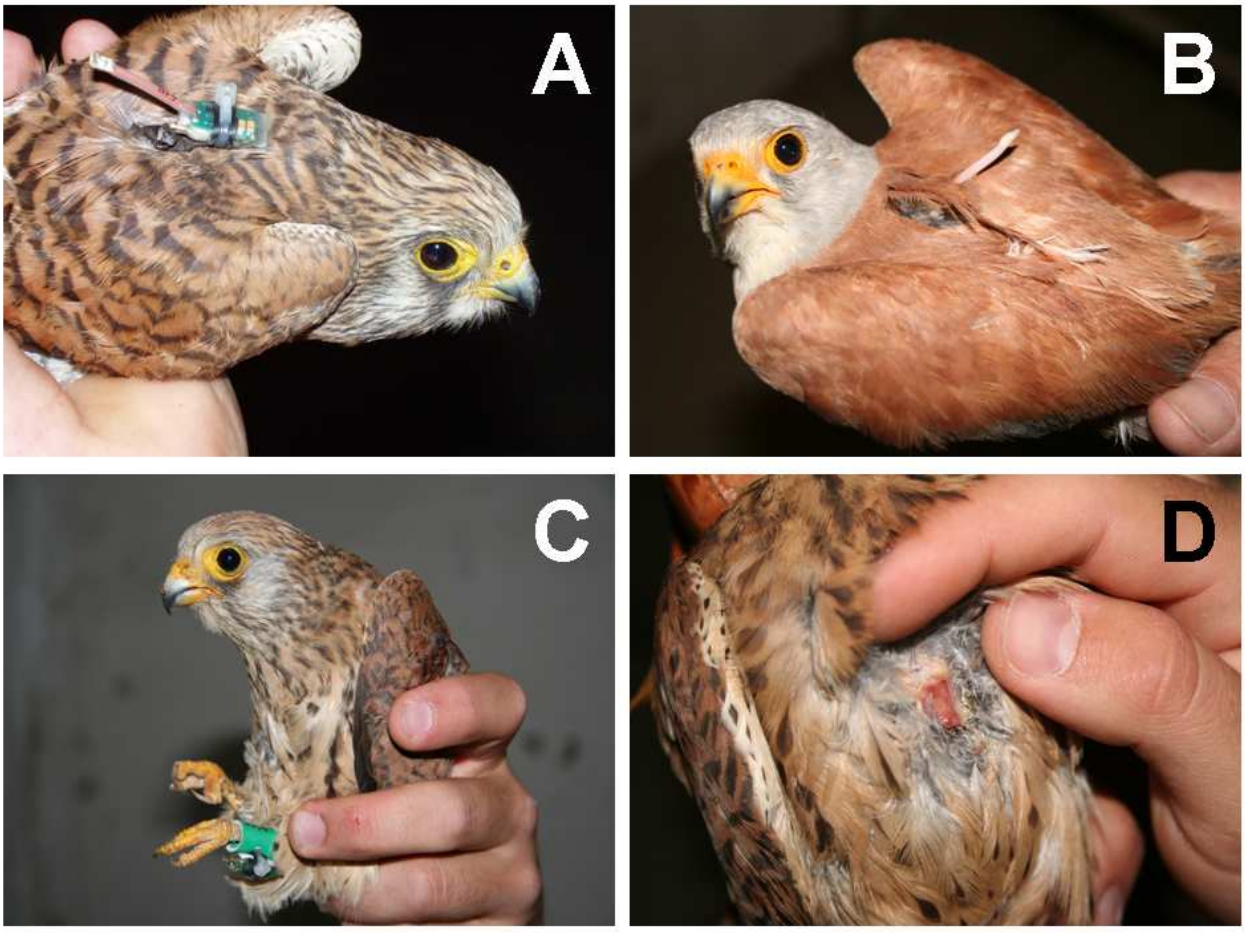


Fig. 2 Geolocator masses (including the whole attachment, e.i. geolocators plus harness/darvic) in relation to body mass of Lesser Kestrels when they were tagged (2007 breeding season; grey boxes) and recaptured (early 2008 breeding season; white boxes). Numbers indicate sample sizes. Dotted internal line, solid line and box boundaries indicate mean, median, and 25% and 75% percentile values, respectively. * indicates classes in which a bird was not measured (see Results).

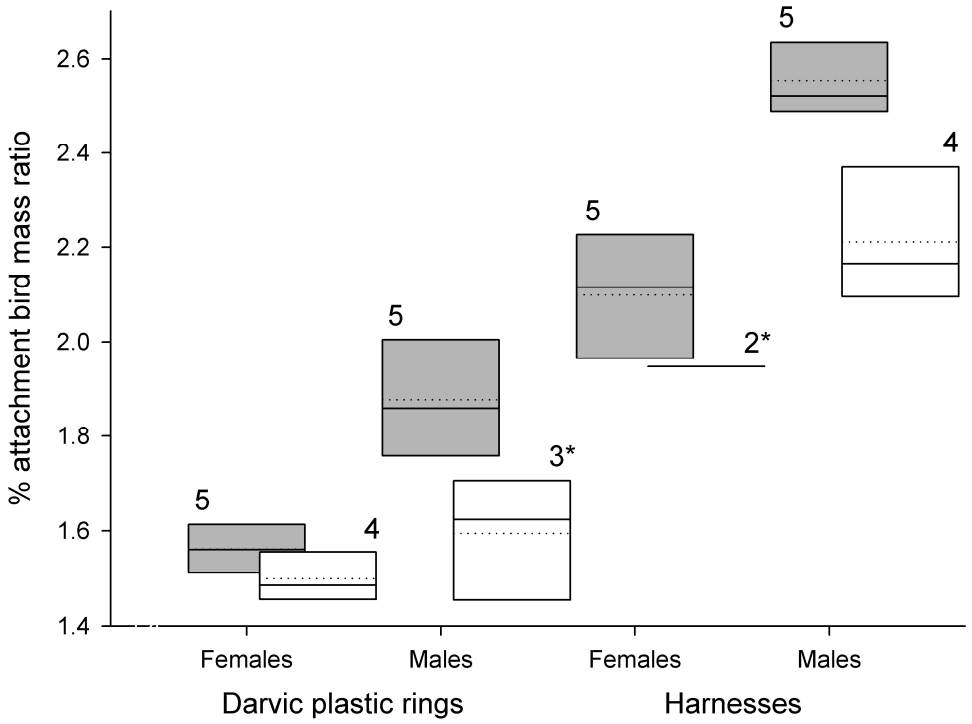
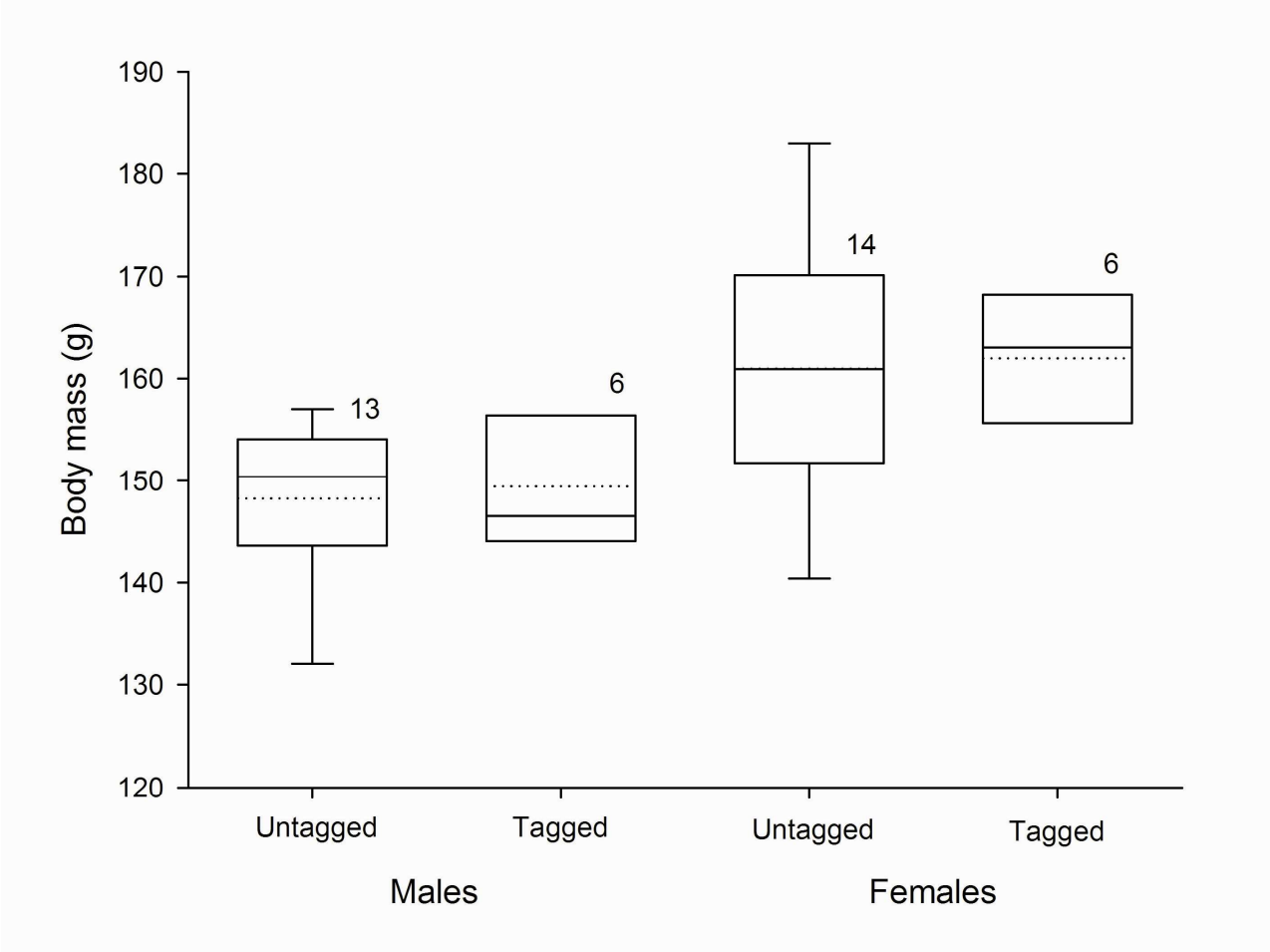


Fig. 3 Body masses of tagged and untagged Lesser Kestrels during the pre-laying period (1 March 2008 - 15 April 2008). Numbers indicate sample sizes. Dotted internal line, solid line, box boundaries and whisker caps indicate mean, median, 25% and 75% percentile values and 10% and 90% percentile values, respectively.



Chapter 3

Using MHC markers to assign the geographic origin of migratory birds: examples from the threatened lesser kestrel

Lesser kestrels foraging on cattle at Senegal. Photo: Philippe Pilard

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Abstract

Gathering knowledge about the migratory routes and wintering areas of threatened populations is fundamental for their successful conservation. Here, we used a non-invasive approach that relies on MHC (Major Histocompatibility Complex) polymorphism to infer the breeding origin of a long-distance migratory bird, the lesser kestrel *Falco naumanni*, in its most important wintering quarters in the Sub-Saharan Africa (Senegal and South Africa). Private alleles support a strong connectivity between wintering Senegalese and western European breeding populations. On the other hand, birds wintering in South Africa were genetically differentiated with respect to western European breeding populations and might therefore gather individuals from the Eastern distribution range. This study demonstrates that, at least at wide continental scales, MHC genes can be powerful intrinsic markers to study migration and migration connectivity, thus adding value to its role in conservation and management.

Introduction

Despite intensive ringing efforts during the last decades and the increasing number of studies in recent years using modern tracking techniques or intrinsic markers such as stable isotopes, trace elements or genetic markers (Webster *et al.* 2002, Hobson 2005, Wink 2006, Coiffait *et al.* 2009), little is known about wintering and stopover sites of many migratory species (Marra *et al.* 2006, Faaborg *et al.* 2010). Given that limiting factors may act on migratory animals in both the breeding and wintering grounds, as well as through the migration routes (Newton 2004), information on population connectivity is crucial for the effective development of conservation and management initiatives of threatened migratory species (Webster *et al.* 2002, Marra *et al.* 2006).

Among popular genetic markers, mitochondrial DNA has been extensively used in phylogeographic studies to unravel spatial patterns of genetic differentiation in the wild. Compared to nuclear DNA, mutations in mitochondrial DNA markers become more rapidly fixed because of a four-times smaller effective population size and the possibility of being more effectively affected by selective sweeps (Ballard & Whitlock 2004). Thus, the utility of mitochondrial DNA markers to resolve evolutionarily significant units and decipher migratory routes is widely recognised (e.g. Banguera-Hinestroza *et al.* 2002, Stefanni & Thorley 2003, Lopes *et al.* 2008, Perego *et al.* 2009). Nevertheless, a single locus approach that can be affected by the co-amplification of nuclear insertions of the mitochondrial genome (i.e. numts; Mindell 1997), genetic introgression and sex-biased dispersal may sometimes complicate and even confound analyses (e.g. Rubinoff & Holland 2005, Hurst & Jiggins 2005). Although mtDNA markers are greatly useful at vast geographical scales, their resolution power at smaller geographical scales has proven unsuccessful in several studies as well (e.g. Lovette *et al.* 2004, Wink *et al.* 2004, Lopes *et al.* 2008). Multilocus genotypes based on polymorphic microsatellite markers have become a popular alternative during the last two decades (e.g. Piry *et al.* 2004, Manel *et al.* 2005). Limited genetic differentiation, mostly attributed to homoplasy and back-mutation of microsatellites, has arisen, however, as an important shortcoming (Queney *et al.* 2001, Boulet & Norris 2006). In fact, several studies have documented low occurrence of private alleles even at vast geographical scales (e.g. Mank & Avise 2003, Alcaide *et al.* 2008).

Despite being widely considered as a classic candidate to reflect local adaptations, studies testing the suitability of the Major Histocompatibility Complex (MHC) to identify the origin of captive or vagrant individuals are surprisingly scarce in the literature. As far as we know, MHC markers have only been used for genetic stock identification of salmons to take

appropriate fishery management decisions (Beacham *et al.* 2001, 2004). The MHC is a multigene family that plays a crucial role during pathogen confrontation and clearance in jawed vertebrates. MHC genes code for cell-surface glycoproteins that bind and present short foreign peptides (antigens) to specialized CD4+ and CD8+ lymphocytes, thus, initiating the development of the adaptive immune response. Extraordinarily high levels of genetic polymorphisms are commonly found within those exons comprising the antigen-binding sites, being large repertoires of alleles maintained by some form of balancing selection (Sommer 2005, Piertney & Oliver 2006, Spurgin & Richardson 2010). The spatio-temporal distribution of MHC variation is expected to reflect pathogen-host co-evolutionary dynamics. Different populations may therefore exhibit contrasting frequencies of the fittest alleles to cope with local pathogen communities. The relative role of neutral evolutionary forces and natural selection on the distribution of MHC diversity nevertheless remains difficult to disentangle in detail (Alcaide 2010).

In this study, we have tested the suitability of MHC markers to infer migratory connectivity in the globally vulnerable lesser kestrel *Falco naumanni* (BirdLife International 2010). This long-distance migratory and colonial falcon breeds in mid-latitudes, from the Iberian Peninsula to China, and winters mainly in the Sub-Saharan Africa (Fig. 1). It has been suggested that populations from different parts of the breeding range tend to remain separated during the winter season, Western-breeding populations migrating to West Africa and Eastern-breeding populations heading to South Africa (Moreau 1972). Although band recoveries, preliminary genetic analyses and tracking of kestrels seem to support this pattern, no conclusive information has been provided so far (see Wink *et al.* 2004; Rodríguez *et al.* 2009, Mihoub *et al.* 2010). Previous analyses of genetic variation at a single MHC class II B gene of the lesser kestrel have revealed extensive genetic polymorphism (>100 alleles) and remarkable patterns of genetic differentiation between European and Asian breeding populations, including a considerable occurrence of private alleles (Alcaide *et al.* 2008, see Supporting Information 1). This pattern contrasted with relatively homogenous distributions of microsatellite alleles but was in agreement with geographic variation at fast evolving mitochondrial DNA sequences (Alcaide *et al.* 2008, see also Wink *et al.* 2004).

Profiting from previous research, our main objective is to infer the breeding origin of the African wintering quarters of lesser kestrels. To this aim, we sampled and MHC-typed naturally shed feathers from two African countries (Senegal and South Africa) known to host thousands of wintering lesser kestrels in large communal roosts (up to 28,600 and 118,000, respectively; LPO 2010, MKP 2010). These numbers roughly represent the

estimated population size of the species in its breeding range (about 140,000 individuals; BirdLife International 2010), and consequently, elucidating its breeding origin is a priority for the conservation of the lesser kestrel.

Materials and Methods

Non-invasive sampling of wintering grounds

Moulted feathers were collected on the ground of two roost sites during a single visit in daylight hours (around 20 January 2007-2009) to avoid disturbing the birds. Feathers were kept in paper envelopes at room temperature until genetic analyses were carried out during the boreal winter of 2009/2010. Sampled roosts were located in Kaolack (14º08´N, 16º05´W, Senegal), and in Phillipstown (30º26´S, 24º28´E, South Africa). These roosts are known to harbour more than 35,000 wintering lesser kestrels (around 28,000 and 7,000 individuals, respectively). Lesser kestrels shared the Senegalese roost with swallow-tailed kites (*Chelictinia riocourii*) and the South African roost with con-generic falcons (red-footed falcon *Falco vespertinus* and Amur falcon *Falco amurensis*) (for more details see LPO 2010, MKP 2010).

DNA extraction, MHC amplification and sequence analyses

DNA extracts were obtained from tips and blood clots of moulted feathers (Horváth *et al.* 2005) according to the HotSHOT protocol (Truett 2006). Information on sampling and DNA extraction from breeding locations is available in Alcaide *et al.* (2008). The second exon of a single and highly polymorphic MHC class II B gene (thereafter referred as *Fana*-DAB locus) was PCR-amplified and sequenced following Alcaide *et al.* (2008). Direct sequencing chromatograms were carefully inspected by eye and edited in BIOEDIT v7.0.5.3 (Hall 1999) and IUPAC (International Union of Pure and Applied Chemistry) nucleotide degenerate codes were introduced for each heterozygous site. MHC diploid genotypes were then resolved into individual haplotypes using the Bayesian PHASE platform (Stephens & Donnelly 2003) implemented in DNASP v5 (Librado & Rozas 2009). For this purpose, we ran unphased genotypes jointly with a database containing more than 100 MHC class II alleles inferred through traditional cloning methods (Alcaide *et al.* 2007, 2008) and also through the investigation of allele segregation patterns from parents to offspring (M. A. unpublished

data). Calculations were carried out over 1,000 iterations, 10 thinning interval and 1,000 burn-in iterations. The information provided by PHASE is valuable because it permits us to assess the presence or absence of informative alleles from Western or Eastern breeding populations (see SI 1). In order to rule out the possibility of sampling the same individual more than once, we discarded those feathers reporting the same MHC genotype (fourteen cases corresponding to four individuals, see SI 2).

Estimates of genetic differentiation between breeding and wintering populations

Genetic differentiation between breeding and wintering grounds was calculated using the nucleotide-sequence-based estimate of genetic differentiation K_{ST} in DNASP (Hudson *et al.* 1992). Furthermore, we calculated an additional genetic measure based on allelic composition between sampling locations (D_{est} –Jost 2008), using the online programme SMOGD v2.6 (Crawford 2010). Both indexes range between 0 (no genetic differentiation; negative values should be treated as 0) and 1 (complete genetic differentiation). Statistical significance was only evaluated for K_{ST} index by permutating haplotypes among samples (9999 permutations). Given the extraordinary extent of genetic polymorphism at the *Fana*-DAB locus, the occurrence of identical alleles in different populations and the very low frequencies of the vast majority of alleles (see Alcaide *et al.* 2008; SI 1 & 2), we did not calculate assignment probabilities for individual birds. Instead, we evaluated whether wintering populations were more genetically related to either European or Asian breeding populations.

Results

Out of the 174 feathers collected in the wintering roosts, 111 (64%) yielded no or weak PCR amplification, ruling out the sequencing of these samples. Feathers collected in 2007 and 2008 showed a lower amplification rate than the feathers collected in 2009 (Likelihood ratio test: $G_2 = 9.32$, $P < 0.009$). No differences in PCR amplification success were detected between wintering roosts (Likelihood ratio test: $G = 1.44$, $P = 0.23$).

Our PHASE-based inferences revealed 41 alleles unreported in the breeding areas. Overall, MHC genotypes permitted us to discriminate up to 27 and 25 genetically distinct individuals in the Senegalese and South African roost, respectively (SI 2). All but three birds (94%) were heterozygous at the *Fana*-DAB locus. The inferring of the gametic phase in

these individuals was highly useful to elucidate the breeding origin of the birds wintering in both geographical areas. Senegalese genotypes reported a high occurrence of alleles previously isolated from European breeding populations (Fig. 2 & SI 2). Twenty-two out of the 34 alleles (64.7%) inferred from the Senegalese roost have been previously isolated in European breeding populations (Fig. 3). In addition, the most abundant alleles within the Senegalese roost (*Fana*2 = 16.6%, *Fana*1 = 7.4% and *Fana*19 = 5.56%) were also among the most abundant alleles in Europe (see SI 1 & 2). None of the alleles isolated in Senegal corresponded to private alleles from the Eastern breeding distribution range. In contrast, only three out 37 South African alleles (8.1%) were previously isolated in European breeding populations. We found no trace of the commonest European alleles, but a high incidence of alleles unreported at the breeding grounds. An important fraction of the alleles isolated in South Africa (5 out 37 different alleles, 13.5 %) were exclusively found in breeding populations from the Eastern distribution range of the species (Fig. 3).

According to our estimates of genetic differentiation (K_{ST} and D_{est}), Western and Central Mediterranean breeding populations were not significantly differentiated with respect to the Senegalese roost but were remarkably differentiated with respect to the South African roost (Table 1). On the other hand, Israeli and Kazakhstani populations showed the highest degree of genetic differentiation when compared to Senegal and the lowest, although still significant, when compared to South Africa (Table 1). Genetic differentiation between the Senegalese and South African roosts was relatively high and significant (K_{ST} = 0.0216, P = 0.0013; D_{est} = 0.928).

Discussion

Our genetic analyses show a compelling genetic resemblance between European breeding populations of lesser kestrels and wintering ones in Senegal. Moreover, our results support previous findings that pinpointed South Africa as an important wintering ground of breeding birds from the Eastern distribution rather than European birds (Wink *et al.* 2004). Besides its relevance for the conservation of the globally vulnerable lesser kestrel, the present study is one of the very few employing MHC markers to decipher the breeding origin of migrating organisms (Beacham *et al.* 2001, 2004) and the first study that has relied on MHC polymorphism to unravel migratory connectivity in birds. It demonstrates the suitability of MHC markers to achieve or complement molecular studies aimed at tracking wildlife in the future. The use of MHC markers in combination with other intrinsic markers

(trace elements, stable isotopes or other genetic markers) might thus significantly enhance our ability to infer migratory routes and trace the origin of captive stock or illegal trade (see Beacham *et al.* 2001, 2004; for similar studies in salmons).

MHC as genetic marker

The *Fana*-DAB locus worked relatively well for non-invasive samples. Importantly, the collection of naturally shed feathers during day hours allowed us to avoid disturbing the birds resting in overcrowded roosts at night. Furthermore, feather collection is a very straightforward task that provides a higher number of samples than non-destructive procedures involving capture and handling of birds (Taberlet *et al.* 1999). As an important pitfall, non-invasive samples usually contain degraded DNA that may result in an increased risk of genotyping errors and allele dropout (Taberlet *et al.* 1999, Segelbacher 2002). From the battery of individuals that we successfully genotyped, heterozygosity levels (0.94) fell in the range documented during previous studies based on the analysis of fresh blood samples (Alcaide *et al.* 2008, 2010). Consequently, we do not expect our results be affected by high rates of allele dropout. Contrary to microsatellites, MHC alleles are identical in size and preferential amplification of small against large alleles is not expected. Furthermore, the relatively low size of polymorphic MHC exons (< 300 bp) make these markers well suited for genetic approaches based on DNA degraded into short fragments (Taberlet *et al.* 1999). The high rates of PCR failure can be nonetheless associated with a high proportion of low quality samples (we could not even observe the blood clot in many of the sampled feathers) and poor storage conditions (Taberlet *et al.* 1999, Segelbacher 2002). In fact, we found significant evidence of higher amplification success for feathers collected in 2009 (with a shorter storage period at room temperature) than those gathered during previous years.

Compared to other nuclear markers, the *Fana*-DAB locus displays higher occurrence of private alleles than microsatellites and patterns of MHC structuring has shown to be as sharp as those revealed by fast evolving mitochondrial DNA sequences (see Wink *et al.* 2004, Alcaide *et al.* 2008). We also experienced higher yields of MHC amplification over mitochondrial markers (authors' unpublished data). Mitochondrial markers have also exhibited methodological problems related to the co-amplification of numts in this and other species (Mindell 1997, Alcaide *et al.* 2008). Although we cannot discard that this fact is due to intrinsic characteristics of the PCR profile, it is known that avian blood is a tissue rich in nuclear DNA (avian erythrocytes are nucleated) but relatively depleted of mitochondrial DNA. Higher yields during PCR amplification can therefore be expected when

targeting nuclear markers on DNA extracts obtained from blood clots. However, working with MHC markers is not easy and several caveats must be highlighted. The characterization of MHC genes in non-model species, the co-amplification of different fragments of the multigene family during single PCR experiments and extensive levels of genetic polymorphism require a considerable set-up before using these markers (reviewed by Babik 2010). However, we have to note that important advances for the amplification and genotyping of MHC genes in non model species have been developed (Babik 2010) and several studies have successfully reported the cross-amplification of MHC fragments across a wide diversity of avian species (e.g. Alcaide *et al.* 2007, 2009, Burri *et al.* 2008, Canal *et al.*, 2010). Even though several studies have documented that MHC markers might be more genetically structured than other nuclear markers in some bird species (see for instance Ekblom *et al.* 2007, Alcaide *et al.* 2008, Loiseau *et al.* 2009), we cannot rule out the lack of spatial patterns of genetic differentiation in others. It is well-known that balancing selection can mitigate the effects of genetic drift (e.g. van Oosterhout *et al.* 2006) and the retention of ancestral polymorphism has been documented, for instance, in passerines (Anmarkrud *et al.* 2010).

The segregation of lesser kestrel breeding populations at wintering range

The genetic data provided in this study agrees with previous assumptions and findings regarding patterns of migratory connectivity in the lesser kestrel (Moreau 1972, Wink *et al.* 2004, Rodríguez *et al.* 2009). A loop migration for Western European breeding kestrels has been hypothesized to cross the Sahara desert in a wide front during the post-nuptial migration and to return through the Western Sahara and/or coastal Africa (Heim de Balsac & Mayaud 1962). Both population size estimates (LPO 2010) and our MHC inferences suggest that an admixture of individuals coming from different European breeding populations may compose the Senegalese roost. This hypothesis would be in agreement with five recent ring recoveries as well (Mihoub *et al.* 2010). On the other hand, the lack of common European MHC alleles in the South African roost, the identification of private alleles from Asian populations, previous research relying on mitochondrial *cytb* sequences (Wink *et al.* 2004) and several ring recoveries (Rodríguez *et al.* 2009) point towards a connection between South African roosts and breeding populations from the Eastern distribution range. The relatively high levels of genetic differentiation between the South African roost and the Asian populations from which we had MHC data could be explained by the congregation of wintering birds from non-sampled and maybe genetically structured

breeding populations from the Eastern distribution range. In fact, the number of birds wintering in South Africa is fivefold that wintering in West Africa (LPO 2010, MKP 2010). Therefore, it would be crucial to elucidate whether kestrels of different roosting flocks originate from the same Eastern breeding areas. In this respect, it is important to notice that an exclusive amino acid motif found at high frequencies in Kazakhstani birds by Alcaide *et al.* (2008), but lacking in European populations, was not found in any sampled feather from the South African roost. Interestingly, this highly informative amino acid motif was found in the two alleles isolated from a museum specimen collected in Kenya in 1915 (deposited at the MCZ collection hosted by Harvard University; ID 78921; authors' unpublished data), suggesting an Asian origin. More research in this region should be encouraged to clarify whether East African regions represent only migratory stopovers or if they are likely to occasionally host large populations of wintering Asiatic birds, especially during those years when the South African wintering population decrease considerably (MKP 2010).

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Tables

Table 1 Estimates of genetic differentiation (K_{ST} and D_{est}) between breeding and wintering populations of lesser kestrels. *P*-values for K_{ST} estimates are showed. Statistically significant values are indicated in bold.

Population	Senegal			South Africa		
	K_{ST}	*P*-value	D_{est}	K_{ST}	*P*-value	D_{est}
SW Spain	0.00227	0.25	-0.04427	0.03406	**<0.001**	0.98819
NE Spain	0.00005	0.40	0.04160	0.03724	**<0.001**	0.96769
France	0.00997	0.07	0.06251	0.05489	**<0.001**	0.92743
Italy	-0.00166	0.54	-0.01332	0.03916	**<0.001**	0.90249
Greece	0.00461	0.19	0.03169	0.03373	**<0.001**	0.97332
Israel	0.01847	**0.010**	0.65273	0.02683	**0.003**	0.60196
Kazakhstan	0.03262	**0.002**	1.00000	0.02310	**0.018**	0.88918

Figures

Fig. 1 Distribution range of lesser kestrel *Falco naumanni* andsampled locations. Dark grey, red and green areas indicate breeding and wintering ranges and partially resident populations of lesser kestrels; respectively (modified from BirdLife International, 2010). SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan; SEN = Senegal; SAF = South Africa; EAF = East Africa (specimens collected in Tanzania and Kenya and deposited at the Museum of Comparative Zoology Harvard University; IDs 133154 and 78921, respectively; see Discussion). Numbers indicate individuals sampled.

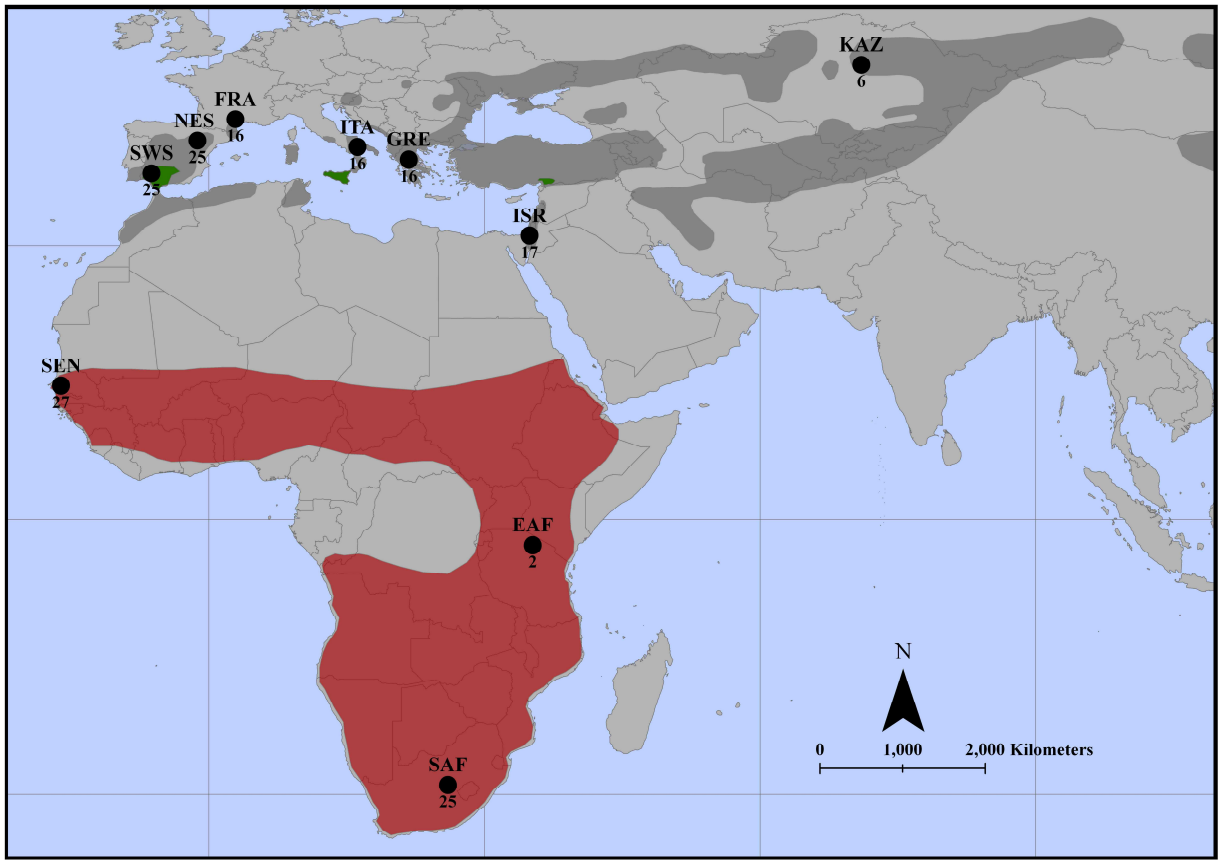


Fig. 2 Number of individuals sampled in the Senegalese and South African roosts showing one (grey) or two alleles (black) present in each breeding populations. SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan.

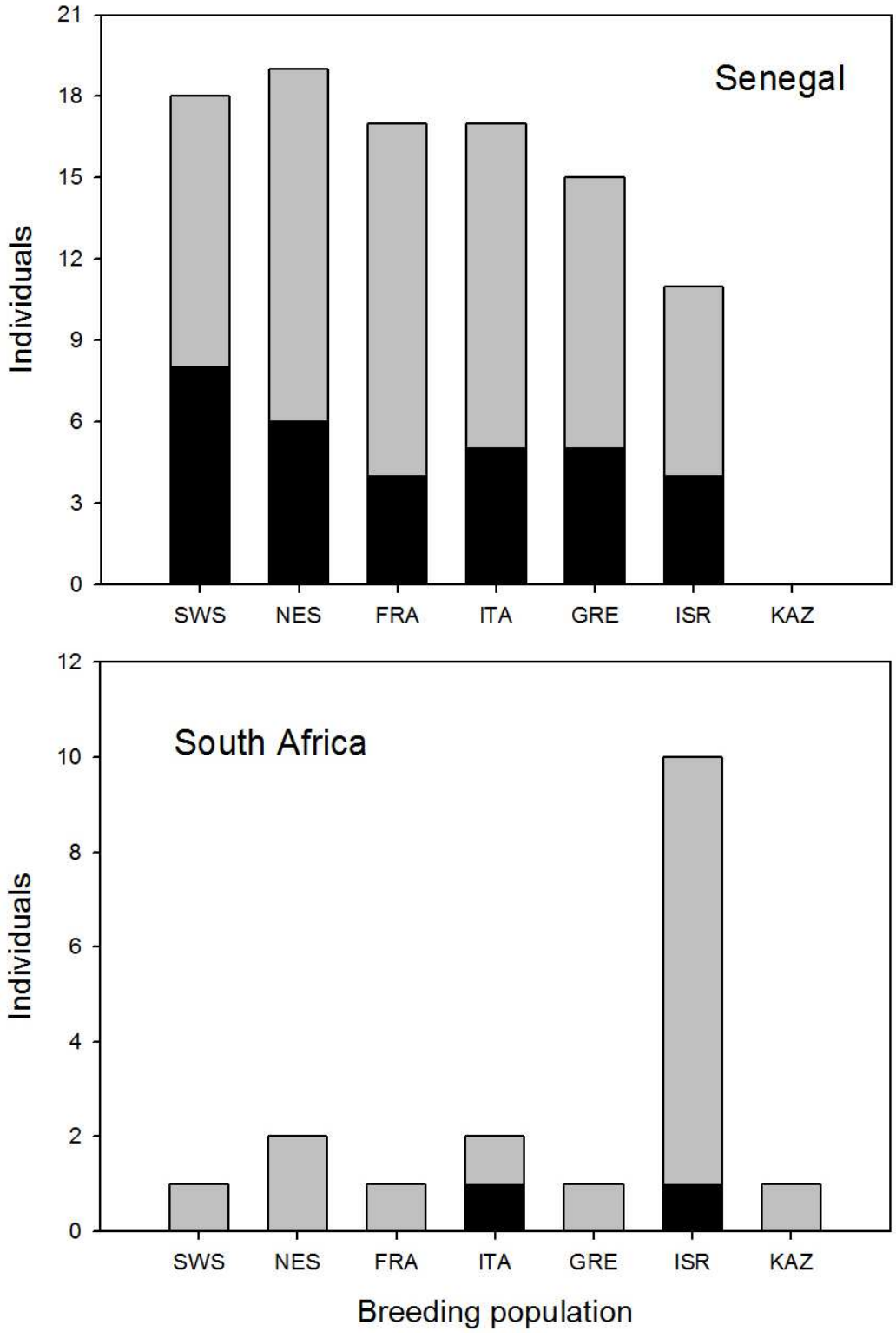
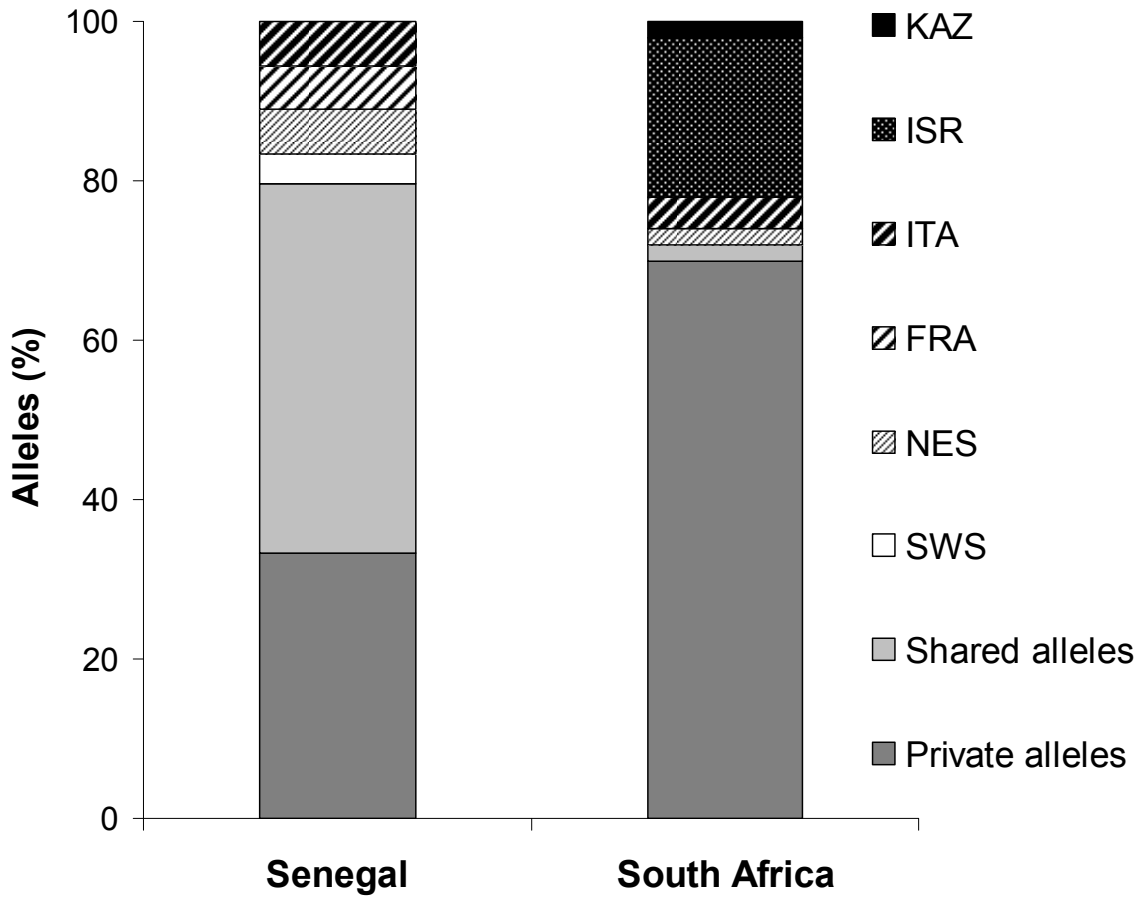


Fig. 3 Origin of alleles found in the wintering areas of lesser kestrels (54 and 50 alleles in Senegal and South Africa, respectively). SWS; NES; FRA; ITA; ISR and KAZ indicate private alleles found in South West Spain; North East Spain; France; Italy; Israel and Kazakhstan; respectively. 'Shared alleles' and 'Private alleles' correspond to alleles present in several breeding areas and alleles not previously isolated in any breeding area, respectively (see SI 1).



Supporting Information

Table S1 Allelic frequencies in breeding and wintering populations of lesser kestrels. Genbank accession numbers are shown. SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan; SEN = Senegal; SAF = South Africa.

Allele	SWS	NES	FRA	ITA	GRE	ISR	KAZ	SEN	SAF	Accesion number
Fana1	0.04000	0.06000	0.12500	0.12500	0.09375	0	0	0.07407	0	EF370839
Fana2	0.20000	0.16000	0.15625	0.18750	0.12500	0.02941	0	0.16667	0	EF370840
Fana3	0.02000	0.02000	0.12500	0.12500	0.03125	0.02941	0	0.01852	0.02000	EF370841
Fana4	0	0.02000	0	0	0	0	0	0.03704	0	EU107670
Fana5	0	0.04000	0	0	0	0	0	0	0	EU107676
Fana6	0	0.04000	0	0	0	0	0	0	0	EU107675
Fana7	0.02000	0	0	0	0.06250	0	0	0.01852	0	EF370855
Fana8	0.04000	0.08000	0.06250	0	0.03125	0	0	0.01852	0	EF370851
Fana9	0.02000	0	0	0	0	0	0	0	0	EF370850
Fana10	0.04000	0	0	0	0	0	0	0	0	EF370842
Fana11	0.02000	0	0	0.03125	0	0	0	0	0	EF370848
Fana12	0.02000	0.02000	0	0	0.03125	0.02941	0	0	0	EF370849
Fana13	0.02000	0	0	0	0	0	0	0	0	EU107731
Fana14	0	0	0	0	0.03125	0	0	0	0	EU107708
Fana15	0	0	0.03125	0	0	0.02941	0	0	0	EU107669
Fana16	0	0	0.03125	0	0	0	0	0	0	EU107716
Fana17	0.02000	0	0	0	0	0	0	0	0	EU107729
Fana18	0.02000	0	0	0	0	0	0	0	0	EF370852
Fana19	0.12000	0.16000	0	0.06250	0	0.05882	0	0.05556	0	EF370854
Fana20	0.02000	0	0	0	0	0	0	0	0	EF370853
Fana21	0.02000	0	0	0	0	0	0	0.01852	0	EU107694
Fana22	0	0	0	0	0	0	0	0.01852	0	EU107700
Fana23	0	0	0.03125	0	0	0	0	0	0	EU107668
Fana24	0	0.02000	0	0	0	0	0	0	0	EU107714
Fana25	0	0.04000	0	0	0	0	0	0	0	EU107715
Fana26	0	0	0	0.03125	0	0	0	0.03704	0	EU107703
Fana27	0	0.02000	0	0	0	0	0	0	0	EF370847
Fana28	0	0	0	0	0	0	0	0	0	EU107673
Fana29	0	0	0.03125	0	0.09375	0	0	0.01852	0	EF370860
Fana30	0.02000	0	0	0	0	0	0	0	0	EU107697
Fana31	0.02000	0	0	0	0.03125	0	0	0.01852	0	EF370858
Fana32	0.04000	0	0	0	0	0	0	0	0	EF370845
Fana33	0	0.04000	0	0	0	0	0	0	0.02000	EF370844
Fana35	0	0	0	0	0	0	0	0.03704	0.02000	EU107704
Fana36	0.02000	0.06000	0	0.06250	0	0.14706	0	0.03704	0	EF370861
Fana37	0	0.02000	0	0	0	0	0	0	0	EU107722

Fana38	0	0	0	0.03125	0	0	0	0	0	EU107684
Fana39	0.04000	0	0	0	0	0	0	0	0	EU107743
Fana40	0	0.02000	0	0	0	0	0	0	0	EU107734
Fana42	0	0	0.03125	0	0	0.11765	0	0	0	EU107682
Fana43	0	0	0	0	0	0.02941	0	0	0	EU107690
Fana44	0	0	0	0	0	0.02941	0.08333	0	0	EU107724
Fana45	0	0.02000	0	0	0	0	0	0	0	EU107702
Fana46	0	0	0	0	0.06250	0	0	0	0	EU107691
Fana47	0	0.02000	0	0	0	0	0	0	0	EU107680
Fana48	0	0	0	0	0.03125	0	0	0	0	EU107733
Fana49	0	0	0	0	0.03125	0	0	0	0	EU107727
Fana50	0	0	0	0	0.06250	0	0	0	0	EU107726
Fana51	0	0	0	0	0.06250	0	0	0	0	EU107725
Fana52	0	0	0	0	0	0	0	0	0	EU107744
Fana53	0	0	0	0	0.03125	0	0	0	0	EF370864
Fana54	0	0	0.03125	0	0	0	0	0	0	EU107732
Fana55	0	0	0.06250	0	0	0	0	0	0	EU107742
Fana56	0	0	0.03125	0	0	0	0	0	0	EU107717
Fana57	0	0	0.03125	0	0	0	0	0	0	EU107707
Fana58	0	0	0	0.03125	0	0	0	0	0	EU107705
Fana59	0	0	0	0	0	0.02941	0	0	0	EU107706
Fana60	0.02000	0	0	0	0	0	0	0.01852	0	EU107740
Fana61	0	0	0	0	0	0.02941	0	0	0	EU107745
Fana62	0	0	0	0	0	0	0	0.01852	0	EU107741
Fana65	0	0	0.06250	0	0	0	0	0.05556	0	EU107686
Fana68	0.02000	0	0	0.03125	0	0	0	0	0	EF370843
Fana69	0	0	0	0	0	0	0.08333	0	0.02000	EU107736
Fana70	0.02000	0	0	0	0	0	0	0	0	EF370859
Fana71	0	0	0	0.03125	0	0	0	0.01852	0.04000	EF370863
Fana72	0	0	0	0	0	0	0	0	0	EU107681
Fana73	0	0	0	0	0	0	0.08333	0	0	EU107701
Fana75	0	0	0	0	0	0.02941	0	0	0	EU107687
Fana76	0	0	0	0	0	0	0.08333	0	0	EU107735
Fana77	0	0	0	0	0	0	0.16667	0	0	EU107712
Fana78	0	0	0	0	0	0	0.08333	0	0	EU107688
Fana79	0	0	0.03125	0	0	0	0	0	0	EU107739
Fana81	0.02000	0.02000	0	0	0	0	0	0	0	EU107730
Fana82	0.02000	0.02000	0	0	0.03125	0	0	0.03704	0	EF370856
Fana83	0	0.04000	0	0	0	0	0	0.01852	0	EF370857
Fana84	0	0	0	0	0.03125	0	0	0	0	EU107720
Fana88	0	0	0	0	0	0	0	0.01852	0	EU107713
Fana89	0	0.02000	0	0	0	0	0	0	0	EU107728
Fana91	0.02000	0.02000	0	0	0	0	0	0	0	EU107693
Fana94	0.02000	0	0	0	0	0	0	0	0	EU107677
Fana97	0	0	0	0	0	0	0.08333	0	0	EU107696
Fana98	0	0	0	0	0	0	0.08333	0	0	EU107689

Fana99	0	0	0	0	0	0	0.08333	0	0	EU107746
Fana100	0	0	0	0	0	0.08824	0	0	0.02000	EU107737
Fana101	0	0	0	0	0	0.02941	0	0	0.08000	EU107699
Fana102	0.02000	0	0	0	0	0	0	0	0	EU107698
Fana103	0.02000	0	0	0	0	0	0	0	0	EU107719
Fana104	0	0	0	0.03125	0	0	0	0	0	EU107718
Fana105	0	0	0	0	0	0	0.08333	0	0	EU107674
Fana106	0	0	0	0	0	0	0.08333	0	0	EU107723
Fana107	0.02000	0	0	0	0	0	0	0	0	EU107738
Fana108	0	0.02000	0	0	0	0	0	0	0	EU107683
Fana109	0.02000	0	0	0	0	0	0	0	0	EU107672
Fana110	0	0	0.06250	0	0	0	0	0	0	HQ402918
Fana111	0	0	0.06250	0	0.03125	0	0	0	0	HQ402919
Fana112	0	0	0	0.06250	0	0	0	0	0	HQ402920
Fana113	0	0	0	0.03125	0	0.02941	0	0	0	HQ402921
Fana114	0	0	0	0.06250	0	0	0	0	0	HQ418344
Fana115	0	0	0	0.03125	0	0	0	0	0	HQ418345
Fana116	0	0	0	0.03125	0.03125	0	0	0	0	HQ418346
Fana117	0	0	0	0	0.03125	0	0	0	0	HQ418347
Fana118	0	0	0	0	0.03125	0	0	0	0	HQ418348
Fana119	0	0	0	0	0	0.08824	0	0	0.04000	HQ418349
Fana120	0	0	0	0	0	0.02941	0	0	0	HQ418350
Fana121	0	0	0	0	0	0.05882	0	0	0.06000	HQ418351
Fana122	0	0	0	0	0	0.05882	0	0	0	HQ418352
Fana123	0	0	0	0	0	0.02941	0	0	0	HQ418353
Fana125	0	0	0	0	0	0	0	0.01852	0	HQ418354
Fana126	0	0	0	0	0	0	0	0.01852	0	HQ418355
Fana127	0	0	0	0	0	0	0	0.01852	0	HQ418356
Fana128	0	0	0	0	0	0	0	0	0.02000	HQ418357
Fana129	0	0	0	0	0	0	0	0	0.02000	HQ418358
Fana130	0	0	0	0	0	0	0	0	0.02000	HQ418359
Fana131	0	0	0	0	0	0	0	0	0.02000	HQ418360
Fana132	0	0	0	0	0	0	0	0	0.04000	HQ418361
Fana133	0	0	0	0	0	0	0	0	0.02000	HQ418362
Fana134	0	0	0	0	0	0	0	0	0.02000	HQ418363
Fana135	0	0	0	0	0	0	0	0	0.02000	HQ418364
Fana136	0	0	0	0	0	0	0	0.01852	0	HQ418365
Fana137	0	0	0	0	0	0	0	0.01852	0	HQ418366
Fana138	0	0	0	0	0	0	0	0.01852	0	HQ418367
Fana139	0	0	0	0	0	0	0	0.01852	0	HQ418368
Fana140	0	0	0	0	0	0	0	0.01852	0	HQ418369
Fana141	0	0	0	0	0	0	0	0.01852	0	HQ418370
Fana142	0	0	0	0	0	0	0	0.01852	0	HQ418371
Fana144	0	0	0	0	0	0	0	0.01852	0	HQ418372
Fana145	0	0	0	0	0	0	0	0.01852	0	HQ418373
Fana146	0	0	0	0	0	0	0	0	0.02000	HQ418374

Fana147	0	0	0	0	0	0	0	0	0.04000	HQ418375
Fana148	0	0	0	0	0	0	0	0	0.04000	HQ418376
Fana149	0	0	0	0	0	0	0	0	0.02000	HQ418377
Fana150	0	0	0	0	0	0	0	0.01852	0	HQ418378
Fana151	0	0	0	0	0	0	0	0	0.02000	HQ418379
Fana152	0	0	0	0	0	0	0	0	0.06000	HQ418380
Fana153	0	0	0	0	0	0	0	0	0.02000	HQ418381
Fana154	0	0	0	0	0	0	0	0	0.02000	HQ418382
Fana155	0	0	0	0	0	0	0	0	0.02000	HQ418383
Fana156	0	0	0	0	0	0	0	0	0.02000	HQ418384
Fana157	0	0	0	0	0	0	0	0	0.02000	HQ418385
Fana158	0	0	0	0	0	0	0	0	0.02000	HQ418386
Fana159	0	0	0	0	0	0	0	0	0.02000	HQ418387
Fana160	0	0	0	0	0	0	0	0	0.02000	HQ418388
Fana161	0	0	0	0	0	0	0	0	0.02000	HQ418389
Fana162	0	0	0	0	0	0	0	0	0.04000	HQ418390
Fana163	0	0	0	0	0	0	0	0	0.02000	HQ418391
Fana164	0	0	0	0	0	0	0	0	0.02000	HQ418392
Fana167	0	0	0	0	0	0	0	0	0.02000	HQ418393
Fana168	0	0	0	0	0	0	0	0	0.02000	HQ418394
Sample size (alleles)	**50**	**50**	**32**	**32**	**32**	**34**	**12**	**54**	**50**	
Nº different alleles	**31**	**24**	**17**	**17**	**21**	**20**	**11**	**34**	**37**	
Nº private alleles	**17**	**14**	**9**	**9**	**10**	**11**	**10**	**16**	**28**	

Table S2 Genotypes of individuals from Senegal and South Africa and presence of alleles in the breeding populations. + = a shared allele with the breeding population; ++ = two shared alleles; SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan.

Individual	Genotype	SWS	NES	FRA	ITA	GRE	ISR	KAZ
Senegal								
2	*Fana8; Fana71*	+	+	+	+	+		
18	*Fana1; Fana125*	+	+	+	+	+		
35	*Fana35; Fana126*							
61; 64	*Fana1; Fana7*	++	+	+	+	++		
69	*Fana1; Fana136*	+	+	+	+	+		
74; 87	*Fana4; Fana137*		+					
75	*Fana65; Fana138*			+				
76	*Fana1; Fana26*	+	+	+	++	+		
78	*Fana2; Fana139*	+	+	+	+	+	+	
79	*Fana26; Fana88*				+			
80	*Fana2; Fana82*	++	++	+	+	++	+	
81	*Fana22; Fana140*							
82	*Fana2; Fana141*	+	+	+	+	+	+	
86	*Fana2; Fana29*	+	+	++	+	++	+	
89	*Fana4; Fana21*	+	+					
91	*Fana36; Fana82*	++	++		+	+	+	
92	*Fana2; Fana142*	+	+	+	+	+	+	
93	*Fana2; Fana3*	++	++	++	++	++	++	
94	*Fana19; Fana36*	++	++		++		++	
99	*Fana2; Fana19*	++	++	+	++	+	++	
10	*Fana31; Fana83*	+	+			+		
102	*Fana19; Fana60*	++	+		+		+	
103	*Fana144; Fana145*							
105	*Fana35; Fana65*			+				
108	*Fana62; Fana127*							
109	*Fana2; Fana2*	++	++	++	++	++	++	
83	*Fana65; Fana150*			+				
South Africa								
114	*Fana69; Fana128*							+
115	*Fana33; Fana129*		+					
122	*Fana130; Fana131*							
123	*Fana121; Fana132*						+	
138	*Fana101; Fana133*						+	
221; 242; 246; 249; 254	*Fana134; Fana135*							
222; 226; 230; 231; 238	*Fana100; Fana35*						+	
152	*Fana119; Fana121*						++	
153	*Fana101; Fana146*						+	
142	*Fana147; Fana147*							
144	*Fana148; Fana148*							
159	*Fana101; Fana149*						+	
140	*Fana119; Fana151*						+	
145	*Fana152; Fana153*							
146	*Fana101; Fana154*						+	

147; 151	*Fana152; Fana155*						
150	*Fana156; Fana157*						
155	*Fana158; Fana159*						
158	*Fana152; Fana160*						
160	*Fana161; Fana162*						
162	*Fana3; Fana162*	+	+	+	+	+	+
167	*Fana163; Fana164*						
112	*Fana71; Fana71*				++		
113	*Fana121; Fana132*						+
116	*Fana167; Fana168*						

Chapter 4

Sampling strategies for accurate computational inferences of gametic phase across highly polymorphic Major Histocompatibility Complex loci

BioEdit panel display showing chromatograms and MHC sequences. Photo: Airam Rodríguez

Alcaide, M., Rodríguez, A. & Negro, J.J. (2011) Sampling strategies for accurate computational inferences of gametic phase across highly polymorphic Major Histocompatibility Complex loci. *BMC Research Notes* 4: 151.

Abstract

Genes of the Major Histocompatibility Complex (MHC) are very popular genetic markers among evolutionary biologists because of their potential role in pathogen confrontation and sexual selection. However, MHC genotyping still remains challenging and time-consuming in spite of substantial methodological advances. Although computational haplotype inference has brought into focus interesting alternatives, high heterozygosity, extensive genetic variation and population admixture are known to cause inaccuracies. We have investigated the role of sample size, genetic polymorphism and genetic structuring on the performance of the popular Bayesian PHASE algorithm. To cover this aim, we took advantage of a large database of known genotypes (using traditional laboratory-based techniques) at single MHC class I (N=56 individuals and 50 alleles) and MHC class II B (N=103 individuals and 62 alleles) loci in the lesser kestrel *Falco naumanni*. Analyses carried out over real MHC genotypes showed that the accuracy of gametic phase reconstruction improved with sample size as a result of the reduction in the allele to individual ratio. We then simulated different data sets introducing variations in this parameter to define an optimal ratio. Our results demonstrate a critical influence of the allele to individual ratio on PHASE performance. We found that a minimum allele to individual ratio (1:2) yielded 100% accuracy for both MHC loci. Sampling effort is therefore a crucial step to obtain reliable MHC haplotype reconstructions and must be accomplished accordingly to the degree of MHC polymorphism. We expect our findings provide a foothold into the design of straightforward and cost-effective genotyping strategies of those MHC loci from which locus-specific primers are available.

Introduction

Highly polymorphic genes of the Major Histocompatibility Complex (MHC) have become very popular molecular markers among evolutionary biologists because of their traditional consideration as 'good genes' involved in pathogen resistance and sexual selection (Sommer 2005, Piertney & Oliver 2006). Despite a plethora of new methods and technical advances (Babik 2010), MHC genotyping still remains challenging and time-consuming. Recently, Bayesian computational inference of gametic phase coupled to Sanger sequencing of PCR amplicons has emerged as a promising alternative (Niu *et al.* 2002, Stephens *et al.* 2001, Stephens & Donnelly 2003, Harrigan *et al.* 2008). These in-silico methods permit researchers to infer how multiple segregating sites are distributed within the same chromosome and are believed to provide haplotype information in a more straightforward and cost-effective way than laboratory-based methods such as cloning, non-denaturing gel electrophoresis and others (Babik 2010). Even though extremely variable MHC loci subjected to the effects of natural selection violate several assumptions of the underlying neutral coalescent theory (Stephens *et al.* 2001), computer packages such as PHASE have shown to perform admirably in many cases (Harrigan *et al.* 2008, Bos *et al.* 2007, Garrick *et al.* 2010). The current version of PHASE, that provides a biologically realistic prior for the distribution of haplotypic frequencies (Stephens & Donnelly 2003), has become one of the most preferred options among evolutionary biologist because of its good performance and the possibility to deal with gaps and polymorphic sites with up to four segregating sites. The accuracy of gametic phase inference has shown to be, however, very sensitive to high heterozygosity, large numbers of alleles and population admixture (e.g. Bos *et al.* 2007). The two first factors are particularly common among MHC genes, a fact that can explain low success rates for particular data sets (Bos *et al.* 2007). In spite of the cost and sample manipulation advantages put forward by these approaches (Babik 2010), only a few studies (e.g. Bos *et al.* 2007, Bettencourt *et al.* 2008) have addressed in detail the relative role of different parameters on PHASE performance when working with highly polymorphic and recombining MHC loci usually exhibiting the genetic hallmarks of balancing and positive selection (i.e. excess of heterozygous sites and non-synonymous substitutions). In this study, we have taken advantage of a large database of MHC class I and class II genotypes built from traditional molecular cloning in the lesser kestrel *Falco naumanni*. Our mains goals were i) test the performance of analytical approaches to haplotype inference in the kestrel MHC, and ii) evaluate the influence of sample size, genetic polymorphism and

genetic structure on the accuracy of computational approaches dealing with phase-unknown diploid genotypes.

Methods

The MHC of the lesser kestrel is well suited for this study because of the specific amplification via the polymerase chain reaction (PCR) of single, highly polymorphic and positively selected MHC class I (exon 3) and MHC class II B (exon 2) loci (Alcaide *et al.* 2007, 2009a). Both loci are 270 base pairs in length and encode for part of the antigen-binding region of MHC class I and MHC class II molecules, respectively. Heterozygosity has been shown to be extremely large in natural populations at both loci (>90%, Alcaide *et al.* 2008, 2010a). A large proportion of the MHC alleles used in this study were isolated during previous studies and many others are derived from ongoing research (Alcaide *et al.* 2007, 2008, 2009a, 2010a, authors unpublished data, see additional file 1). The handling and sampling of the birds was done in accordance with Spanish laws concerning animal welfare, and under permission of the different National Governments.

We created two data sets, one for each particular MHC locus. Overall, we gathered the known genotypes of 56 heterozygous birds at the MHC class I locus and 103 heterozygous individuals at the MHC class II B locus. Even though homozygous individuals at both loci have been reported we decided to exclude them from our data sets as a means to create the most challenging scenario during the evaluation of the performance of the PHASE algorithm. The two data sets encompassed 50 MHC class I and 62 MHC class II alleles, respectively (see Table 1 and additional files 1 and 2). For the MHC class I data set, only four and two alleles, respectively, showed frequencies beyond 5% and 10%. For the MHC class II data set, only seven and three alleles, respectively, showed frequencies higher than 5% and 10%. The two data sets also represented different degrees of genetic structuring. In the case of the MHC class I, individual genotypes were obtained from birds captured in Spain, France, Italy, Greece and Israel and restrictions in gene flow are thus expected (see Alcaide *et al.* 2008). Individuals of the MHC class II data set were exclusively sampled from Spain, which can be essentially considered as a panmictic population according to both neutral and adaptive genetic data (Alcaide *et al.* 2008, 2009b). We created different sample subsets containing 8, 15, 30 or 45 individuals from the MHC class I data set. In the case of the MHC class II, sample subsets were composed of 8, 15, 30, 45, 60 or 75 individuals. Five groups of individuals were randomly sub-sampled for each sample size.

The knowledge of the real genotypes beforehand permitted us to generate those ambiguous DNA sequences resulting from the overlapping of the two alleles isolated per individual at each MHC locus (see additional file 2). These consensus DNA sequences were generated using the software BioEdit (Hall 1999). With this information, we performed a reverse approach through which analytical approaches relying on ambiguous diploid data would be validated with respect to the genotypes inferred using traditional laboratory-based techniques. Bayesian computational inference of MHC gametic phase was performed using the popular, user-friendly PHASE module implemented in the software DNAsp ver 5.0 (Librado & Rozas 2009). Calculations were carried out over 1,000 iterations, 10 thinning interval and 1,000 burn-in iterations and considering a model that accounted for recombination. All the advanced options available for the algorithm were settled as default. PHASE accuracy was measured as the percentage of correctly assigned alleles. We concluded that the two alleles at each locus were correctly inferred when all nucleotide positions matched perfectly to those previously revealed by laboratory-based methods. To verify the identity of each allele, we took advantage of the output window provided by default by the software DNAsp 5.0 and we exported the alignment as a FASTA file subsequently handled in BioEdit.

Results

Our results show a remarkable influence of sample size on the accuracy of haplotypic inference using PHASE (Fig. 1). For both MHC loci, average accuracy improved along with sample size. The number of alleles not correctly inferred was proportional to the number of genotypes. This is due to fact that when PHASE failed to infer one of the two alleles from a given genotype it incorrectly inferred the sequence of the other allele as well (i.e. one or a few segregating sites where switched between the two alleles). Overall, PHASE errors were related to the incorrect calling of one or a few segregating sites, and at least, PHASE seemed to do rather well when inferring the allelic lineage. The increase of PHASE accuracy along with sample size can be attributed to the reduction in the ratio between the number of alleles occurring in the sample set and the number of individuals comprising that particular sample set. To get deeper insights about the influence of the allele to individual ratio, we created simulated data that introduced variations in this parameter. In these simulations, we altered the allele-to-individual ratio for a sample size of 25 individuals and 40 individuals for the MHC class I and class II locus, respectively. The simulated genotypes

were heterozygous in all cases and we tried to distribute allele frequencies as equally as possible. Only in the case of the simulation of seven class I alleles (see Fig. 2) we repeated 4 out the 25 heterozygous genotypes used in the same sample set. In the remaining cases, the number of possible combinations of alleles in heterozygous form was larger than sample size (i.e. N=25 and N=40 for the MHC class I and class II data set, respectively). We added 15 MHC class II B alleles isolated during previous studies (Alcaide *et al.* 2008, 2010a) in order to gather the 80 alleles needed for the 2:1 allele individual ratio. The manipulation of the allele-to-individual ratio had a dramatic influence on PHASE performance (Fig. 2). For instance, the accuracy of computational inferences of MHC haplotypes was very poor when the number of alleles was twice than that of individuals. Nonetheless, the performance of PHASE consistently increased along with the reduction of the allele to individual ratio. From the comparison between the two MHC data sets, and regardless of the degree of genetic structuring within the geographic area individuals were sampled from, we suggest a ratio allele to individual starting at (1:2).

The main objective of this study was to provide useful information regarding the number of individuals to be sampled, given a particular degree of genetic polymorphism, to computationally infer the gametic phase of MHC genes with reliability. Starting from a "worst-case" scenario similar to that used in our simulations (i.e. no occurrence of homozygous individuals and with homogenous distributions of allele frequencies), we recommend a first exploratory view of 25-30 individuals. Although PHASE can miscall nucleotides during the reconstruction of haplotypes, our experience suggests that the overall number of alleles inferred is not very different from the actual number. Depending on the number of alleles inferred by PHASE, researchers might add more individuals until the allele to individual ratio reaches at least the 1:2 threshold. Sampling strategies must therefore be designed according to the extent of MHC polymorphisms found within a particular study population. Hopefully, researchers might find homozygous genotype*s* or genotypes comprised by alleles just differing in one or a few nucleotides during sampling. This might be indeed very useful regarding the verification of the set of inferred alleles. It is also advisable to ground-truth the data set by performing molecular cloning in a selected number of individuals. Molecular cloning, however, is extremely prone to report false polymorphisms and therefore, it is important to contrast cloned alleles with direct sequencing chromatograms. Special caveats should be considered in the case of synonymous diploid genotypes (i.e. different combinations of alleles can generate the same direct sequencing chromatogram). However, careful examination of our allele repertoire suggests that these cases are rare in kestrels (<1% of possible genotypes). The additional

aid of technologies such as conformational polymorphism analyses (e.g. Alcaide *et al.* 2010b) may nonetheless become very useful to resolve these particular cases. Researchers must pay special attention to generate high-quality direct sequencing chromatograms to minimize the risk of miscall double peaks. In this respect, the performance and location of sequencing primers as well as bi-directional sequencing must be carefully addressed. Finally, it is important to bear in mind that these approaches can only be achieved when locus-specific primers are available (Bettinotti *et al.* 2003, Hughes *et al.* 2008, this study). That said, our better genomic knowledge of the MHC in both model and non-model species (e.g. Worley *et al.* 2008, Cloutier *et al.* 2011) forecasts an encouraging future in this respect.

Acknowledgments

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Tables

Table 1 Polymorphisms statistics at the kestrel MHC class I and class II data sets used in this study. This table compiles the number of alleles (Na), the number of variable sites (S), the total number of mutations (Eta), mean nucleotide diversity per site (π) and the average number of nucleotide differences between alleles (*k*).

Locus	**Na**	**S**	**Eta**	**π**	***k***
MHC class I	50	37	41	0.030	8.45
MHC class II	62	60	75	0.078	21.04

Figures

Fig. 1 Influence of the number of individuals analysed on the performance (percentage of alleles correctly assigned; bars go with primary Y-axis) of Bayesian reconstructions of MHC haplotypes using the PHASE algorithm and the allele-to-individual ratio (open circles go with secondary Y-axis). Standard deviations for each parameter are indicated.

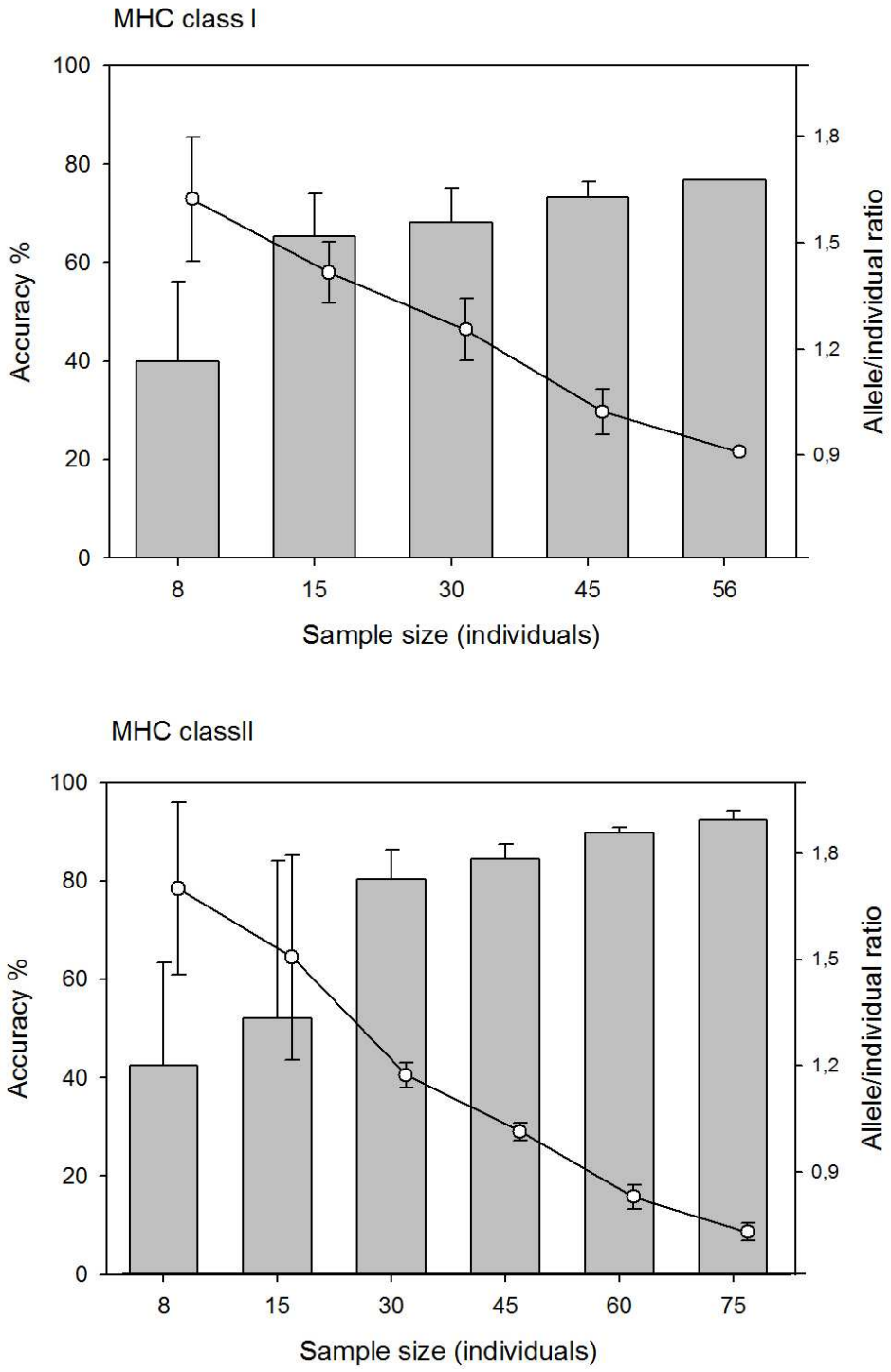
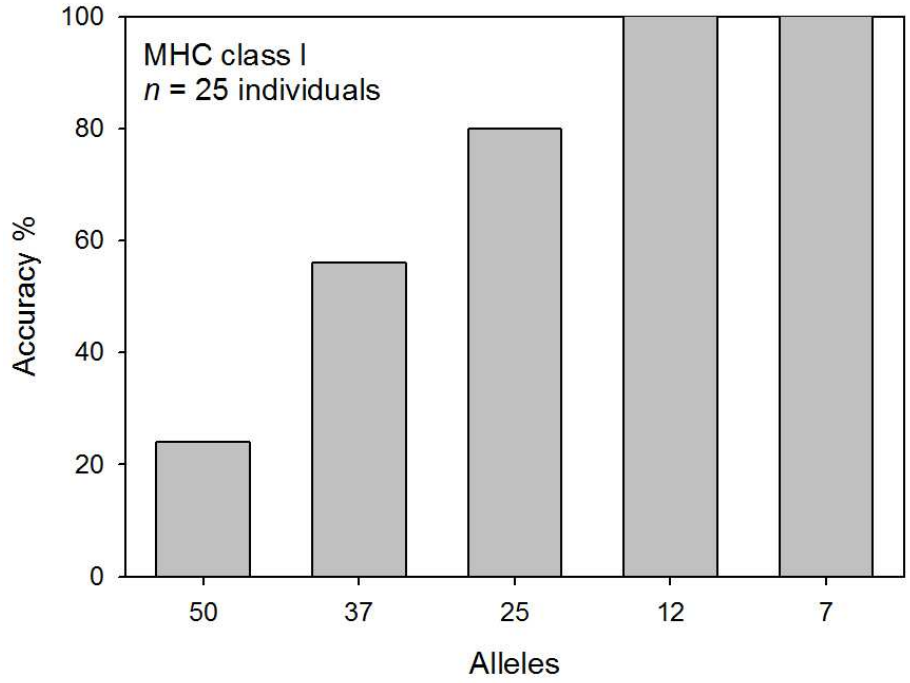
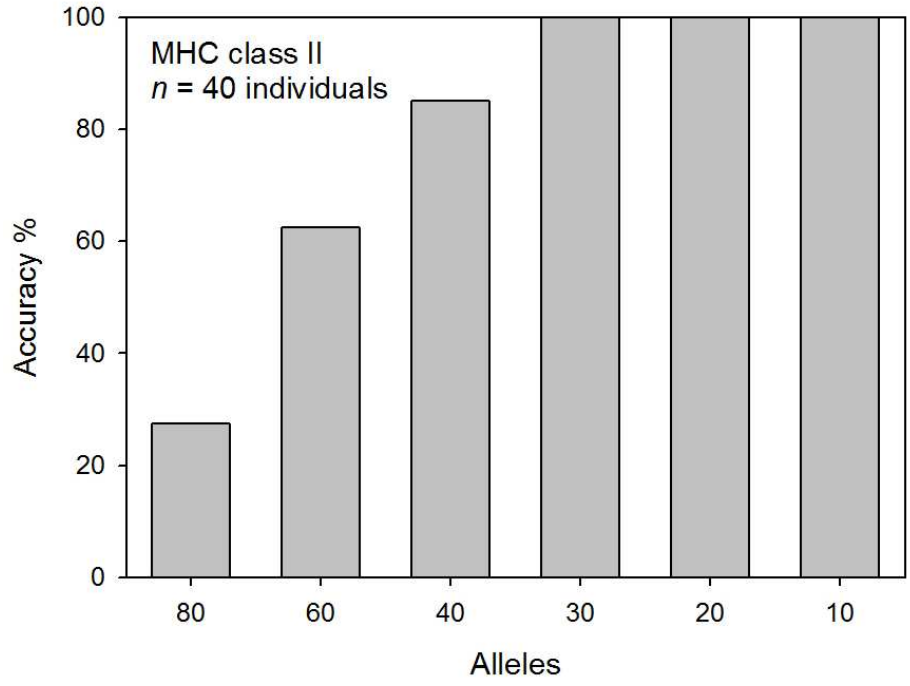


Fig. 2 Influence of the allele-to-individual ratio on the performance of Bayesian reconstructions of MHC haplotypes using the PHASE algorithm. We altered the number of alleles for a sample size of 25 individuals for the MHC class I and 40 individuals for the MHC class II data sets. Standard deviations for each parameter are indicated.





Supporting information

Table 1 MHC class I and MHC class II B genotypes resolved by traditional laboratory-based methods during previous studies [11-14] and ongoing research by the authors. GenBank accession numbers for the MHC alleles of the lesser kestrel *Falco naumanni* are shown.

Genotype	MHC Class I		MHC Class II	
1	*Fana10*	*Fana11*	*Fana35*	*Fana36*
2	*Fana2*	*Fana13*	*Fana62*	*Fana83*
3	*Fana2*	*Fana11*	*Fana1*	*Fana19*
4	*Fana8*	*Fana18*	*Fana19*	*Fana41*
5	*Fana12*	*Fana33*	*Fana29*	*Fana40*
6	*Fana4*	*Fana11*	*Fana4*	*Fana12*
7	*Fana11*	*Fana19*	*Fana2*	*Fana91*
8	*Fana14*	*Fana17*	*Fana2*	*Fana71*
9	*Fana2*	*Fana41*	*Fana17*	*Fana7*
10	*Fana20*	*Fana47*	*Fana1*	*Fana2*
11	*Fana1*	*Fana35*	*Fana34*	*Fana35*
12	*Fana11*	*Fana13*	*Fana2*	*Fana26*
13	*Fana10*	*Fana29*	*Fana3*	*Fana79*
14	*Fana2*	*Fana34*	*Fana60*	*Fana10*
15	*Fana4*	*Fana10*	*Fana19*	*Fana47*
16	*Fana6*	*Fana11*	*Fana2*	*Fana8*
17	*Fana2*	*Fana16*	*Fana1*	*Fana33*
18	*Fana3*	*Fana10*	*Fana3*	*Fana91*
19	*Fana11*	*Fana52*	*Fana26*	*Fana29*
20	*Fana9*	*Fana19*	*Fana60*	*Fana36*
21	*Fana2*	*Fana35*	*Fana8*	*Fana39*
22	*Fana6*	*Fana36*	*Fana39*	*Fana68*
23	*Fana11*	*Fana59*	*Fana2*	*Fana19*
24	*Fana2*	*Fana48*	*Fana11*	*Fana12*
25	*Fana27*	*Fana60*	*Fana12*	*Fana19*
26	*Fana11*	*Fana20*	*Fana2*	*Fana7*
27	*Fana15*	*Fana38*	*Fana40*	*Fana31*
28	*Fana6*	*Fana40*	*Fana2*	*Fana83*
29	*Fana9*	*Fana42*	*Fana2*	*Fana65*
30	*Fana2*	*Fana24*	*Fana1*	*Fana8*
31	*Fana7*	*Fana10*	*Fana111*	*Fana82*
32	*Fana17*	*Fana20*	*Fana19*	*Fana48*
33	*Fana24*	*Fana25*	*Fana26*	*Fana82*
34	*Fana1*	*Fana5*	*Fana79*	*Fana80*
35	*Fana2*	*Fana6*	*Fana29*	*Fana51*
36	*Fana7*	*Fana11*	*Fana31*	*Fana50*
37	*Fana13*	*Fana28*	*Fana2*	*Fana87*
38	*Fana38*	*Fana57*	*Fana3*	*Fana7*
39	*Fana2*	*Fana20*	*Fana2*	*Fana40*

40	*Fana34*	*Fana62*	*Fana7*	*Fana21*
41	*Fana2*	*Fana27*	*Fana1*	*Fana3*
42	*Fana2*	*Fana45*	*Fana1*	*Fana85*
43	*Fana20*	*Fana35*	*Fana9*	*Fana1*
44	*Fana7*	*Fana19*	*Fana19*	*Fana42*
45	*Fana19*	*Fana63*	*Fana36*	*Fana72*
46	*Fana11*	*Fana46*	*Fana3*	*Fana35*
47	*Fana17*	*Fana47*	*Fana2*	*Fana53*
48	*Fana32*	*Fana47*	*Fana2*	*Fana63*
49	*Fana2*	*Fana7*	*Fana29*	*Fana36*
50	*Fana20*	*Fana50*	*Fana1*	*Fana27*
51	*Fana11*	*Fana35*	*Fana19*	*Fana67*
52	*Fana20*	*Fana22*	*Fana19*	*Fana52*
53	*Fana11*	*Fana23*	*Fana19*	*Fana68*
54	*Fana10*	*Fana30*	*Fana19*	*Fana87*
55	*Fana27*	*Fana31*	*Fana23*	*Fana2*
56	*Fana6*	*Fana39*	*Fana12*	*Fana37*
57			*Fana2*	*Fana4*
58			*Fana1*	*Fana72*
59			*Fana1*	*Fana12*
60			*Fana12*	*Fana33*
61			*Fana82*	*Fana83*
62			*Fana2*	*Fana37*
63			*Fana8*	*Fana19*
64			*Fana68*	*Fana75*
65			*Fana9*	*Fana36*
66			*Fana29*	*Fana107*
67			*Fana2*	*Fana89*
68			*Fana111*	*Fana2*
69			*Fana2*	*Fana57*
70			*Fana1*	*Fana36*
71			*Fana2*	*Fana3*
72			*Fana31*	*Fana62*
73			*Fana36*	*Fana83*
74			*Fana47*	*Fana60*
75			*Fana1*	*Fana34*
76			*Fana35*	*Fana1*
77			*Fana8*	*Fana10*
78			*Fana81*	*Fana94*
79			*Fana2*	*Fana10*
80			*Fana10*	*Fana32*
81			*Fana1*	*Fana21*
82			*Fana19*	*Fana20*
83			*Fana32*	*Fana70*
84			*Fana2*	*Fana31*
85			*Fana1*	*Fana18*
86			*Fana9*	*Fana17*
87			*Fana19*	*Fana83*
88			*Fana1*	*Fana25*

89	*Fana4*	*Fana90*
90	*Fana2*	*Fana60*
91	*Fana34*	*Fana41*
92	*Fana31*	*Fana68*
93	*Fana87*	*Fana109*
94	*Fana19*	*Fana34*
95	*Fana52*	*Fana26*
96	*Fana8*	*Fana40*
97	*Fana19*	*Fana62*
98	*Fana2*	*Fana36*
99	*Fana2*	*Fana62*
100	*Fana82*	*Fana2*
101	*Fana3*	*Fana82*
102	*Fana19*	*Fana36*
103	*Fana111*	*Fana57*

Genbank accession numbers

MHC class I locuss

*Fana*1: EU120671
*Fana*2: EU120672
*Fana*3: EU120675
*Fana*4: EU120667
*Fana*5: JF831086
*Fana*6: EU120676
*Fana*7: EU120668
*Fana*8: EU120669
*Fana*9: EU120670
*Fana*10: EU120665
*Fana*11: EU120664
*Fana*12: EU120674
*Fana*13: EU120666
*Fana*14: JF831087
*Fana*15: EU120673
*Fana*16: EU120677
*Fana*17: EU120678
*Fana*18: EU120679
*Fana*19: JF831088
*Fana*20: JF831089
*Fana*22: JF831090
*Fana*23: JF831091
*Fana*24: JF831092
*Fana*25: JF831093
*Fana*27: JF831094
*Fana*28: JF831095
*Fana*29: JF831096
*Fana*30: JF831097
*Fana*31: JF831098
*Fana*32: JF831101
*Fana*33: JF831099
*Fana*34: JF831102
*Fana*35: JF831100
*Fana*36: JF831105
*Fana*38: JF831104
*Fana*39: JF831103
*Fana*40: JF831110
*Fana*41: JF831108
*Fana*42: JF831111
*Fana*45: JF831109
*Fana*46: JF831112
*Fana*47: JF831107
*Fana*48: JF831113
*Fana*50: JF831114
*Fana*52: JF831115
*Fana*57: JF831116
*Fana*59: JF831117
*Fana*60: JF831118
*Fana*62: JF831119
*Fana*63: JF831120

MHC class II B locus

*Fana*1: EF370839
*Fana*2: EF370840
*Fana*3: EF370841
*Fana*4: EU107670
*Fana*7: EF370855
*Fana*8: EF370851
*Fana*9: EF370850
*Fana*10: EF370842
*Fana*11: EF370848
*Fana*12: EF370849
*Fana*17: EU107729
*Fana*18: EF370852
*Fana*19: EF370854
*Fana*20: EF370853
*Fana*21: EF370846
*Fana*23: EU107668
*Fana*25: EU107715
*Fana*26: EU107703
*Fana*27: EF370847
*Fana*29: EF370860
*Fana*31: EF370858
*Fana*32: EF370845
*Fana*33: EF370844
*Fana*34: EU107671
*Fana*35: EU107704
*Fana*36: EF370861
*Fana*37: EU107722
*Fana*39: EU107743
*Fana*40: EU107734
*Fana*41: EU107721
*Fana*42: EU107682
*Fana*47: EU107680
*Fana*48: EU107733
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*Fana*60: EU107740
*Fana*62: EU107741
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Chapter 5

Sources of variation for nutritional condition indices of the plasma of migratory lesser kestrels in the breeding grounds

Lesser kestrel nestling feeding on a bush-cricket. Photo: Pepe Antolín

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Abstract

Although published information on reference values for biochemical parameters in birds of prey has increased during the last years, little is known on their sources of variation. We used an insectivorous and small migratory raptor species, the lesser kestrel *Falco naumanni*, as a model. We looked for sources of variation of nutritional biochemical parameters (i.e. triglycerides, cholesterol, uric acid and urea) of both nestlings and adults. Reference values indicated that, as a rule, lesser kestrel showed more elevated triglycerides, urea and uric acid levels than other raptors. All analyzed factors except gender and presence/absence of a geolocator (i.e. year, colony, sampling time, body mass, laying date and capture date) reached significance for at least one biochemical parameter. In the morning, we found an important postprandial increase in the concentration of all biochemical parameters in nestlings, and uric acid and urea levels in adults. A positive relationship was detected between triglycerides and body mass in nestlings and adults. Although we did not find differences between blood biochemical parameters of the oldest and youngest chick of each brood, we found that cholesterol levels were lower in nestlings from larger broods. Coloration of tarsi (measured as brightness) was related to triglycerides and urea levels of nestlings and adults, respectively. The feeding habits of lesser kestrel probably explain the different levels and patterns of variation of metabolites in comparison to more carnivorous raptors eating mammals or birds.

Introduction

During long-term fasting in endotherm vertebrates, three different physiological phases based on changes in body mass and plasma biochemistry can be distinguished (see review in McCue 2010). The first phase of fasting is characterized by a severe body-mass loss as well as a decrease of the levels of uric acid and urea in plasma. During the second phase, stored lipids are used as an energy source, but the protein catabolism residues (urea and uric acid) maintain a low concentration. In the third phase (prolonged fasting), levels of glucose in blood drop dramatically and uric acid and urea reach high values due to the use of structural proteins as an energy resource (Bauchinger & Biebach 2001). Finally, animals suffer a severe body mass loss approaching death. During the three fasting phases, triglycerides steadily decrease (Jenni-Eiermann & Jenni 1998, Alonso-Alvarez & Ferrer 2001).

Despite an increase of studies publishing reference values of free-living raptors during the last years (e.g. Stein *et al.* 1998, Casado *et al.* 2002, Hanauska-Brown *et al.* 2003, Sarasola *et al.* 2004, Mealey *et al.* 2004, Limiñana *et al.* 2009, Hernández & Margalida 2010), most of the studies focusing on blood biochemistry have been conducted on captive birds. However, these have non-natural diets, regular access to food, and are forced to live in relative small enclosures in comparison with the natural home ranges, and thus, blood parameters might vary with respect to free-ranging animals (Dobado-Berrios *et al.* 1998, Balbontín & Ferrer 2002, Villegas *et al.* 2002). Therefore, reference values for biochemical parameters of wild individuals are invaluable for ecophysiologists studying free-ranging birds (e.g. nutritional status, presence of diseases or exposure to pollutants; e.g. Artacho *et al.* 2007), and may also help the managers of rehabilitation centers or captive programs to keep birds in conditions as similar as possible to the wild ones. Furthermore, the majority of studies focusing on blood biochemistry have been conducted in large raptors (e.g. eagles or vultures) which ingest relatively large amounts of food at one time and endure longer fasting periods than smaller species. Therefore, feeding habits could influence the interpretation of some biochemistry values, especially those related to prolonged fasting periods, such as urea and uric acid. In large raptors, high values of these parameters have invariably been interpreted as indicators of low nutritional condition (Ferrer 1994, Ferrer & Dobado-Berrios 1998, Balbontín & Ferrer 2005).

The lesser kestrel *Falco naumanni* is one of the smallest European raptor species (along with the Merlin *Falco columbarius* and the Red-Footed Falcon *Falco vespertinus*). Its diet is basically composed of insects (i.e., grasshoppers, beetles, crickets), but it also feeds

on small mammals (Rodríguez *et al.* 2010, Pérez-Granados 2010 and references therein). In contrast to larger raptors, the lesser kestrel typically eats several times per day due to the small size of its prey. Starvation is the main cause of nestling mortality (Negro 1997), suggesting they are not well adapted to long fasting periods. This migratory falcon's breeding range spans from China to the Iberian peninsula and its wintering grounds are located in sub-Saharian Africa (Rodríguez *et al.* 2009a, 2011). Since the 1950s, its populations have suffered a severe decline (estimated at more than 30% of the world population), leading to its current vulnerable status (BirdLife International 2011). As a consequence, numerous breeding programs have been put in place for reintroduction purposes (Pomarol 1993, Alcaide *et al.* 2010). Therefore, information presented here on sources of variation of nutritional biochemical parameters is valuable to assess condition of the captive stock, which in Spain only numbers several hundred pairs in at least five different breeding centers.

We have analyzed four biochemical parameters related to fat (triglycerides and cholesterol) and protein metabolism (uric acid and urea; see McCue 2010), in adult and nestling lesser kestrels. These parameters have shown to be related to the physiological and nutritional state of birds (Jenni-Eiermann & Jenni 1998, Alonso-Alvarez & Ferrer 2001, Alonso-Alvarez *et al.* 2002a, 2002b, Sarasola & Negro 2004). Our aim is, first, to report reference values of selected plasma biochemical parameters that have been previously reported as nutritional condition indices in larger and mainly vertebrate-eating birds of prey. Second, to examine the influence of different factors, such as sex, colony, year, sampling time, body mass, brood size, capture date –for adults– and laying date –for nestlings–, on nutritional markers. Third, to evaluate differences of biochemical parameters between the eldest and the younger nestlings from the same brood, as in the lesser kestrel death due to starvation typically affects sequentially the youngest and smallest individuals in the brood. Thus, we hypothesize that youngest nestlings will show lower triglyceride and cholesterol levels and higher nitrogenous waste levels (urea and uric acid) as a consequence of protein catabolism. Last, we aim to determine the relationships between certain phenotypic traits (i.e. characteristics of plumage –tail band width– and bare parts coloration –brightness and chroma of tarsi) and blood biochemical parameters. We expect that these traits will be negatively correlated with nutritional condition.

Materials and Methods

Study area and trapping procedures

Adults and nestlings were captured during three consecutive breeding seasons (2007-2009) in two neighboring urban colonies distant 10 km, the cereal silo of La Palma del Condado (37º23'N, 6º33'W) and the Purificación church of Manzanilla (37º23'N, 6º25'W), Huelva province, southern Spain (for a detailed description of surrounding habitat see Rodríguez *et al.* 2006). Adults were captured from March to July at their nest cavities. Nestlings were sampled at the nest at the age of 24.0 ± 3.4 days (± SD). All birds were captured by hand and several body measurements were recorded (body mass, eighth primary, tail and wing length -the latter two only for adults). Body mass was measured with an electronic balance to the nearest 0.1 g and length measurements were done with a ruler to the nearest 1 mm. The width of the black terminal tail band was measured with a caliper at the raquis of the top central tail feather.

Color measurements

Coloration of the tarsi was measured using a Minolta 2600 spectrometer (Minolta, Osaka, Japan), which uses a high-energy xenon flash illumination and a dual-40-element silicon photodiode array (e.g. Negro *et al.* 2006). Automatic white calibration was performed before all measurements. Color was evaluated as the averaged reflectance over the wave length interval (360-740 nm) for brightness and as the difference between the maximum and minimum reflectance divided by the averaged reflectance for chroma (Anderson & Prager 2006).

Blood sampling and plasma biochemistry

All birds were bled from the brachial vein (approximately 0.5 ml) between 9:00 and 15:00 h to minimize expected variations caused by circadian rhythms of the biochemical parameters (García-Rodríguez *et al.* 1987, Pérez-Rodríguez *et al.* 2008). The blood was collected in a heparinized tube and kept in a cooler until centrifugation. Blood was centrifuged (10 min; 4500 rpm; 4 ºC), and the plasma and the cellular fraction were stored at -20 ºC until analyses were conducted. Plasma was analyzed for triglycerides, cholesterol, urea, and uric acid using a Screen Point autoanaliser (Hospitex Diagnostics, Sesto Fiorentino, Italy), and commercial kits (Biolabo Labs, Maizy, France). Plasma biochemical analyses were performed by Wildvets S.L.P. (Seville, Spain; Rodríguez *et al.* 2009). To assess

repeatability (Lessells & Boag 1987), two measurements of each biochemical parameter were taken from 99 randomly selected samples (30, 44 and 25 samples from 2007, 2008 and 2009, respectively; eleven adults –six females– and 88 nestlings –44 females). Coefficients of repeatability varied between 0.95 and 0.99. For analyses, we employed the mean values of the two readings of these repeated samples.

Sex determination

As the Lesser Kestrel shows a strong sexual dichromatism, adults were visually sexed according to plumage characteristics. However, gender determination of nestlings is sometimes difficult (Rodríguez *et al.* 2005). In this case, the cellular fraction was used as a source of DNA. We determined sex by PCR amplification of CHD genes using primers 2550F and 2718R (Fridolfsson & Ellegren 1999).

Statistical analyses

First, an ANOVA was employed to test differences in the plasma metabolite concentrations of nestlings and adults. As data coming from siblings in a same nest are not independent and some adults were sampled twice or more times, we used mean values for each brood and each adult individual, respectively. Second, we used general linear mixed models to explain the variations in blood biochemical parameters. Nestlings' models included sex, year and colony as factors, and sampling time, body mass and laying date as covariates. To avoid the non-independence of data from siblings, brood identity was included as a random effect. As some adult birds were fitted with geolocators during the 2007-2008 winter and some biochemical parameters of nestlings were affected by this procedure (see Rodríguez *et al.* 2009b), we included an additional fixed factor to control for this source of variation (0 = non geolocator-tagged bird or nestling without tagged parents, 1 = geolocator-tagged bird or nestling with at least one tagged parent). Adults' models included sex, year and age (yearlings or older than 1 year) as factors, and sampling time, body mass and date of capture as covariates. To avoid pseudoreplication, identity was included as a random effect given that some adult individuals were captured in different breeding seasons. Third, to assess the effect of brood size on biochemical parameters of nestlings, we ran models including the significant terms of our previous analyses and an ordinal variable (number of nestlings). In addition, we built general linear mixed models including the sibling order (the younger and the eldest), brood size and their interaction as fixed factors and brood identity

as a random factor. We introduced the interaction because differences between the younger and the eldest nestlings may be more apparent in larger brood sizes. The eighth primary feather of the wing has been used to estimate age of nestlings (Negro 1997), thus, we assumed the eldest and youngest siblings in a brood showed the longest and the shortest eighth primary, respectively. To test the relationship between brood size and the nutritional condition of adults, we selected those adults captured during chick rearing activities (after June 10th). Given that this dataset contained a smaller number of individuals (n = 39), we used Pearson correlation analyses including breeding success (number of fledglings) and each biochemical parameter. Fourth, to evaluate the relationships between coloration phenotypic traits and biochemical parameters, we built models including the significant terms in the previous analyses. Each phenotypic trait (tail band width, chroma, and brightness) was included individually.

Because variable responses were not normally distributed, they were log or square-root transformed. *F*-ratio was used to test the significance of fixed effects in our full models (no backward or stepwise procedures were employed). Statistical analyses were conducted using JMP v.9 package. In some cases, the amount of plasma obtained was insufficient to assay all the parameters. For this reason, sample sizes are not uniform.

Results

Reference values

A total of 334 nestling, belonging to 112 broods, and 104 adult lesser kestrels were studied. Twenty eight adult birds were sampled two or three times, increasing the sample size for adults to 135. Excepting for urea levels, mean values for all biochemical parameters were significantly different between nestlings and adults, although ranges overlapped (Table 1).

Factors affecting blood parameters

The main factor affecting the blood biochemical parameters of nestlings was sampling time (Table 2), which reached significance for all parameters analyzed. A positive relationship was detected through the morning for all biochemical parameters (Fig. 1). Cholesterol, uric acid and urea levels were affected by year of sampling, indicating that it is a crucial factor in the nutritional status of lesser kestrel nestlings. Triglyceride levels were also affected by

colony, and they showed a positive relationship with body mass and laying date. Last, levels of urea increased with laying date. The geolocator tagging effect was not significant for any parameter. Models explained from 26.9 to 55.7 % of the total variation in the blood parameters (Table 2). Brood identity (random factor) explained from 6.1 to 38.5 % of the total variation (Table 2).

For adults, levels of triglycerides varied between years and showed a positive relationship with body mass (Fig. 2). Cholesterol concentration was not explained by any independent variable measured in this study. Uric acid and Urea were affected by sampling time, and additionally by date of capture (positive relationship) and by year of sampling, respectively (Table 2). Models explained from 20.5 to 46.8 % of the total variation in the blood parameters (Table 2). The random factor (individual identity) explained low percentages of the total variation (from 2.7 to 10 %).

Effect of brood size on biochemical parameters

Models including the significant terms listed in Table 2 for each biochemical parameters and the number of nestlings (as an ordinal variable) indicated that cholesterol was negatively affected by brood size in nestlings (F = 3.88; d.f. = 4, 120.1; P = 0.005; Fig. 3A). Number of nestlings tended to be positively related to triglycerides (F = 2.07; d.f. = 4, 126.8; P = 0.087), but in a second analysis excluding the single nestlings, the relationship reached significance (F = 2.47; d.f. = 3, 94.5; P = 0.047; see Fig. 3B). Number of siblings was not related to uric acid nor urea (F = 1.18; d.f. = 4, 113.3; P = 0.322 and F = 0.16; d.f. = 4, 103.5; P = 0.956, respectively. No significant differences were found between the younger and eldest nestlings for any biochemical parameter (all P-values > 0.125) or the interaction between hatching order and brood size (all P-values > 0.163). In adults, only urea levels were correlated with number of fledglings (r = 0.325; P = 0.049, Fig. 4).

Correlates of biochemical parameters and phenotypic traits

Tarsi brightness was the single phenotypic trait which correlated with some blood biochemical parameter. Negative relationships were observed between brightness and triglyceride levels, and brightness and urea levels for nestlings and adults, respectively (see Table 3).

Discussion

Reference values

We provide for the first time reference values for four plasma biochemical parameters indicative of nutritional condition for free-living nestling and adult lesser kestrels. Nearly all ranges of plasma metabolites for nestlings and adults overlapped with those described for free-living larger raptorial birds (Dobado-Berrios *et al.* 1998, Ferrer & Dobado-Berrios 1998, Stein *et al.* 1998, van Wyk *et al.* 1998, Bowerman *et al.* 2000, Casado *et al.* 2002, Villegas *et al.* 2002, Balbontín & Ferrer 2002, Hanauska-Brown *et al.* 2003, Mealey *et al.* 2004, Sarasola *et al.* 2004, Limiñana *et al.* 2009, Hernández & Margalida 2010). However, we consistently detected higher mean values of triglycerides in lesser kestrel than in the above cited studies on larger raptors. Given that plasma triglyceride levels decline after short periods of fasting (Jenni-Eiermann & Jenni 1997), these differences might be explained by the higher feeding frequency of the lesser kestrel in comparison with larger raptors, which endure longer fasting periods, sometimes more than a week, due to unpredictability of their food. In this sense, adult lesser kestrels need 3.1-9 min to acquire a prey item during hunting activities (Donázar *et al.* 1993, Tella *et al.* 1998) and the prey delivery rates to nestlings vary between 2.04-3.98 items per hour (Tella *et al.* 1996), although under food shortage conditions, prey delivery rates can notably decrease (Negro *et al.* 2000, Rodríguez *et al.* 2006).

While reported in fewer studies, urea and uric acid also were lower in some raptor species (Lavin *et al.* 1992, Stein *et al.* 1998, Bowerman *et al.* 2000, Villegas *et al.* 2002, Balbontín & Ferrer 2002, Mealey *et al.* 2004, Sarasola *et al.* 2004, Limiñana *et al.* 2009, Hernández & Margalida 2010), or in non-raptorial species such as the greater flamingo *Phoenicopterus roseus* or the white stork *Ciconia ciconia* (Amat *et al.* 2007, Jerzak *et al.* 2010). Plasma urea and uric acid are protein catabolism residues and good indicators of low nutritional condition because protein catabolism is active during the last phase of fasting (McCue 2010). However, high levels of these parameters can be induced by protein-rich diets (Jenni-Eiermann & Jenni 1998). Although a non negligible part is sequestered in indigestible exoskeleton, grasshoppers contain higher levels of proteins than mammals or birds (Bird *et al.* 1982). Thus, we think differences reported here are a consequence of the lesser kestrel mainly insectivorous diet (based on Ortopthera; e.g. Rodríguez *et al.* 2010) more than to the effect of starvation (i.e. protein catabolism; Jenni-Eiermann & Jenni 1998, McCue 2010) given the high levels of triglycerides.

Factors affecting blood parameters

The important effect of sampling time has been reported in several studies using captive birds (García Rodríguez *et al.* 1987, Ferrer *et al.* 1994, Pérez-Rodríguez *et al.* 2008), but, as far as we know, it has been only described in a published field study for triglycerides and uric acid levels in adult passerines (Jenni-Eiearmann & Jenni 1997). We found an increase in the concentration of all four biochemical parameters with sampling time as daytime advanced (triglycerides, cholesterol, uric acid and urea in nestlings, and uric acid and urea in adults; Fig. 1). The increase in the values of blood biochemical parameters detected in nestlings seems to be related to the increase of feeding rates in the morning. Thus, during the first daytime hours we sampled nestlings that had fasted overnight (at least for the nine nighttime hours), but that were subsequently fed by parents during the morning. In adults, the same pattern was found for uric acid and urea levels (Fig. 1), and it may be due to energy cost incurred in food provisioning given that an increase for triglyceride and cholesterol levels was not observed. The positive relationship between plasma uric acid concentration and date of capture, which suggests an important energy cost of breeding duties, also supports this idea. However, we cannot discard that the same hypothesis suggested for nestlings can be acting on adults, i.e. a postprandial increase in the morning.

Contrary to other studies focused on larger raptors (Casado *et al.* 2002, Sarasola *et al.* 2004) or other bird species (e.g. Artacho *et al.* 2007, Jerzak *et al.* 2010), no differences between sexes were found for plasma concentration of the four analyzed parameters, despite the sexual size dimorphism of lesser kestrel and the mate-feeding by males to their female mates during the breeding period (Donázar *et al.* 1992). However, no sexual differences have been detected in free-living nestlings of other sexual size dimorphic raptors such as the Spanish imperial eagle *Aquila adalberti*, the Bonelli's eagle *Aquila fasciata* or the Montagu's harrier *Circus pygargus* (Ferrer & Dobado-Berrios 1998, Balbontín & Ferrer 2002, Limiñana *et al.* 2009) or in free-living adults of the American kestrel *Falco sparverius* or the northern goshawk *Accipiter gentilis* (Stein *et al.* 1998, Hanauska-Brown *et al.* 2003).

Inter-annual variation seems to be another important factor explaining differences in the biochemical parameters of lesser kestrel nestlings, as it reached significance for cholesterol, uric acid and urea. For these three parameters, lowest values were detected in 2007 and the highest ones in 2008 (data not shown). Also for adults, year of sampling reached significance for triglyceride and urea levels, although the pattern for triglycerides

was opposite to nestlings (data not shown). These annual differences could be related to quantitative and qualitative differences in prey abundance as a consequence of annual weather conditions, which have a strong effect on breeding success (Rodríguez & Bustamante 2003). However, we detected a decrease in the breeding success during the studied years (2007, 2008 and 2009 reaching productivity values of 3.3, 3.2 and 2.5 fledglings per successful nest, respectively), that suggests the involvement of multiple factors, apart from diet.

Triglycerides level seems to be one of the best indicators of fasting in field studies given its interspecific consistency (Jenni-Eiearmann & Jenni 1997). Positive relationships between triglycerides concentration and body mass (or body condition) have been described in passerines, raptors and pelecaniformes (Jenni-Eiearmann & Jenni 1998, Sarasola *et al.* 2004, Villegas *et al.* 2004). Our data also indicate a relationship between triglyceride and condition, both in nestlings and adults (Fig. 2).

We found a positive relationship between urea levels and laying date of nestlings (Table 2). The same pattern was found in Spanish imperial eagle nestlings, but not in Bonelli's eagle nestlings (Balbontín & Ferrer 2005), and the former was interpreted as earlier broods having better-nourished nestlings (Ferrer 1994). However, triglyceride levels of nestlings also increased with the laying date in our study (Table 2), suggesting that a poorer diet is not the actual explanation, but more probably a change in the diet (Rodríguez *et al.* 2010).

Effect of brood size on biochemical parameters

For nestlings, we found a negative relationship between cholesterol and brood size (Fig 3). The differences were pronounced between extreme values of brood size, thus nestlings from nests with brood size equal to 1 showed the highest values and nestlings in broods with 5 nestlings showed the lowest levels. In the case of triglycerides and brood size, a high variability was observed in the single nestlings, but a clear positive relationship was found for nests with 2-5 nestlings. Although we do not have data to prove it, different type of prey delivered to nestlings by parents according to brood size may explain the observed trends.

Spanish imperial eagle nestlings from nests with larger brood sizes were better nourished (Ferrer 1994) and Bonelli's eagle nestlings from nests with two siblings showed a poorer condition than single nestlings during a year of adverse environmental circumstances (heavy rainfall; Balbontín & Ferrer 2005). However, these conclusions were obtained based on urea and uric acid plasma levels. Interestingly, we did not find any

relationship with these two parameters, and there were not clear differences between the oldest and youngest siblings. This suggests that nestlings were equally fed by parents independently of their age, probably as a consequence of the high habitat quality and productivity of our colonies. The two colonies studied here (Silo and Purificación) showed the best habitat quality in comparison with four other colonies, and their habitat quality is high enough for population stability, according to a modeling approach based on prey abundance and prey size (see Rodríguez *et al.* 2006).

The relationship between urea levels and breeding success of adults may be explained by the work load of reproduction duties (Fig. 4). It has been experimentally demonstrated that kestrels adjust the provisioning rates according to brood size (Dawson & Bortolotti 2003). Thus, the parents rearing a high number of nestlings have high feeding provisioning rates, reducing time for themselves. This would fit with the negative correlation between brightness and urea levels (see below).

Correlates of biochemical parameters and phenotypic traits

Melanin-based traits are static signals and may provide information on nutritional status at the time when feathers were growing. This may explain the lack of correlation between tail band width and biochemical parameters, which are indicative of short-term nutritional status (Jenni-Eiearmann & Jenni 1998). However, colored patches of bare skin are a reflection of recent physiological events, indicating the current physiological condition of the individual (e.g. Negro *et al.* 2006). High carotenoid plasma concentrations and brilliant coloration of fleshy parts have been reported as indicators of quality in kestrels (Bostrom & Ritchison 2006, Casagrande *et al.* 2006, Dawson & Bortolotti 2006, Vergara & Fargallo 2011). Carotenoid pigments are responsible of yellow-orange coloration of kestrel tarsi (Bortolotti *et al.* 1996, Casagrande *et al.* 2009), and important physiological modulators, but these pigments can not be synthesized *de novo* by birds. Thus, a trade-off between the show and health functions have been suggested (Negro *et al.* 1998).

Brightness is inversely proportional to pigment concentration (Andersson & Prager 2006). The negative relationships between brightness and triglyceride levels in nestlings suggest that the nestlings better nourished (higher triglyceride levels) had the higher carotenoid concentration in tarsi (lower brightness). In contrast, the negative relationship between brightness and urea levels in adults may be a consequence of higher work loads of high quality individuals (low brightness) rearing higher number of nestlings (see above).

Conclusions

The small size (atypical for the majority of raptor species studied so far for biochemical parameters) and its insectivorous diet suggest lesser kestrel may be more alike, ecologically speaking, to a medium sized passerine, such a shrike, than to a typical raptor. The high feeding frequency seems to be responsible for the general higher values of triglycerides, while the insectivorous diet may explain the higher uric acid and urea levels observed here. Also the increase of feeding rates during the morning may be the explanation for the increase of metabolite concentration, which is a key factor for future studies sampling at different times.

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Tables

Table 1 Blood chemistry values and results of ANOVA tests for nestling and adult Lesser Kestrels. All biochemical parameters are expressed as mg/dl. Mean, SD and range are absolute values, i.e. they have not been transformed. ANOVA tests were conducted using the mean values of nestlings from the same nest and mean values of adults sampled twice or more times (see text).

	Nestlings			Adults					
Parameter	*n*	Mean ± SD	Range	*n*	Mean ± SD	Range	*F*	*d.f.*	*P*
Triglycerides [a]	332	301.4 ± 203.8	[34.6 , 1038]	135	257.2 ± 255.3	[53.2 , 1388.7]	15.23	1, 211	**< 0.001**
Cholesterol [a]	326	216.1 ± 65.1	[87.1 , 476.6]	132	275.7 ± 91.7	[87.9 , 789.6]	30.17	1, 209	**< 0.001**
Uric acid [b]	320	16.8 ± 6.4	[3.6 , 38.5]	121	15.1 ± 7.6	[3.9 , 34.0]	4.98	1, 203	**0.027**
Urea [b]	317	17.2 ± 9.1	[5.1 , 59.5]	124	16.0 ± 9.4	[0.7 , 47.5]	3.95	1, 204	**0.052**

[a] Variable log transformed. [b] Variable square-root transformed.

Table 2 Summary of fixed effects and the overall variability explained (R^2) by the general linear mixed models on biochemical parameters of nestling and adult lesser kestrels. All response variables were log transformed, excepting uric acid (nestling model) and urea (adult model) which were square-root transformed.

	Triglycerides		Cholesterol		Uric acid		Urea	
	Estimate ± SE	*P*	Estimate ± SE	*P*	Estimate ± SE	*P*	Estimate ± SE	*P*
Nestlings								
Intercept	**1.009 ± 0.290**	**< 0.001**	**2.035 ± 0.167**	**< 0.001**	1.011 ± 0.966	0.297	0.378 ± 0.241	0.119
Sex	-	0.102	-	0.087	-	0.398	-	0.187
Female	0.023 ± 0.001	-	0.011 ± 0.006	-	0.036 ± 0.042		0.013 ± 0.010	
Year	-	0.611	-	**< 0.001**	-	**< 0.001**	-	**< 0.001**
2007	0.022 ± 0.025	-	**-0.050 ± 0.015**	-	**-0.294 ± 0.082**	-	**-0.113 ± 0.020**	-
2008	0.010 ± 0.022	-	**0.032 ± 0.013**	-	**0.282 ± 0.074**	-	**0.061 ± 0.018**	-
Colony	-	**0.005**	-	0.515	-	0.912	-	0.534
Silo	**-0.053 ± 0.019**	-	-0.008 ± 0.012	-	-0.007 ± 0.062	-	-0.010 ± 0.016	-
Geolocator	-	0.111	-	0.825	-	0.195	-	0.287
Untagged	-0.035 ± 0.022	-	-0.003 ± 0.014	-	-0.096 ± 0.074	-	-0.020 ± 0.018	-
Time of day	**0.056 ± 0.011**	**< 0.001**	**0.017 ± 0.007**	**0.011**	**0.142 ± 0.037**	**< 0.001**	**0.029 ± 0.009**	**0.002**
Body mass	**0.002 ± 0.001**	**0.012**	0.000 ± 0.001	0.822	0.003 ± 0.003	0.269	-0.000 ± 0.001	0.826
Laying date	**0.004 ± 0.002**	**0.024**	0.001 ± 0.001	0.522	0.008 ± 0.005	0.148	**0.004 ± 0.001**	**0.002**
Brood identity (%)	6.1	-	38.4	-	17.3	-	22.2	-
R^2 (%)	26.9	-	55.7	-	36.1	-	45.8	-
Adults								
Intercept	**0.678 ± 0.323**	**0.038**	**2.527 ± 0.183**	**< 0.001**	0.094 ± 0.265	0.778	0.564 ± 1.483	0.705
Sex	-	0.478	-	0.264	-	0.750	-	0.751

Female	-0.023 ± 0.032	-	-0.020 ± 0.018	-	0.008 ± 0.026	-	0.046 ± 0.143	-
Year	-	**< 0.001**	-	0.157	-	0.506	-	**0.031**
2007	**0.185 ± 0.042**	-	-0.031 ± 0.024	-	-0.035 ± 0.035	-	**-0.344 ± 0.198**	-
2008	**-0.056 ± 0.043**	-	0.046 ± 0.024	-	0.040 ± 0.036	-	**0.511 ± 0.191**	-
Age	-	0.168	-	0.262	-	0.405	-	0.514
	0.048 ± 0.034	-	0.022 ± 0.019	-	0.023 ± 0.028	-	0.105 ± 0.161	-
Time of day	0.028 ± 0.017	0.103	-0.003 ± 0.010	0.797	**0.049 ± 0.014**	**< 0.001**	**0.173 ± 0.078**	**0.028**
Body mass	**0.009 ± 0.002**	**< 0.001**	-0.000 ± 0.001	0.947	0.001 ± 0.002	0.357	0.003 ± 0.008	0.722
Capture date	0.000 ± 0.001	0.796	-0.001 ± 0.001	0.199	**0.002 ± 0.001**	**0.006**	0.006 ± 0.004	0.162
Identity (%)	10.0	-	7.0	-	8.1	-	2.7	-
R^2 (%)	46.8	-	20.5	-	38.4	-	26.1	-

Table 3 Results of General Linear Mixed models for phenotypic traits introducing significant terms of Table 2 and brood identity (for nestlings) and individual identity (for adults) as random factors.

Variable response	Variable independent	Estimate ± SE	*F*	*d.f.*	*P*
Nestlings					
Triglycerides [a]	Tail Band Width	0.00038 ± 0.00385	0.01	1, 275.9	0.921
	Chroma	0.10854 ± 0.13807	0.91	1, 278.3	0.341
	Brightness	- 0.01157 ± 0.00487	5.65	1, 269.2	**0.018**
Cholesterol [a]	Tail Band Width	0.00055 ± 0.00169	0.10	1, 285.8	0.745
	Chroma	- 0.08467 ± 0.05869	2.08	1, 301.3	0.150
	Brightness	0.00041 ± 0.00216	0.03	1, 302.8	0.851
Uric acid [b]	Tail Band Width	-0.00758 ± 0.01195	0.40	1, 273.1	0.527
	Chroma	0.01123 ± 0.39688	0.00	1, 276.4	0.977
	Brightness	- 0.01057 ± 0.01484	0.50	1, 281.1	0.477
Urea [a]	Tail Band Width	0.00114 ± 0.00295	0.14	1, 272.4	0.699
	Chroma	- 0.16672 ± 0.09967	2.79	1, 281.3	0.096
	Brightness	0.00339 ± 0.00371	0.83	1, 290.8	0.361
Adults					
Triglycerides [a]	Tail Band Width	0.00665 ± 0.00415	2.56	1, 104.3	0.112
	Chroma	0.04139 ± 0.14824	0.07	1, 120.6	0.781
	Brightness	- 0.01163 ± 0.00731	2.52	1, 123.7	0.115
Cholesterol [a]	Tail Band Width	0.00217 ± 0.00206	1.11	1, 100.7	0.294
	Chroma	- 0.03074 ± 0.07727	0.15	1, 126.4	0.691
	Brightness	0.00216 ± 0.00400	0.29	1, 126.7	0.591
Uric acid [a]	Tail Band Width	0.00221 ± 0.00301	0.53	1, 82.12	0.466
	Chroma	0.06414 ± 0.12143	0.25	1, 104.9	0.599
	Brightness	- 0.00511 ± 0.00604	0.71	1, 113.3	0.399
Urea [b]	Tail Band Width	- 0.00183 ± 0.01705	0.01	1, 66.86	0.915
	Chroma	0.15205 ± 0.65867	0.05	1, 112.6	0.818
	Brightness	- 0.06485 ± 0.03199	4.11	1, 112.2	**0.045**

[a] Variable log transformed. [b] Variable square-root transformed.

Figures

Fig. 1 Relationship between biochemical parameters of nestling (filled circles) and adult (open circles) lesser kestrels and sampling time. Least square means (± SE) from saturated models from Table 2 are shown.

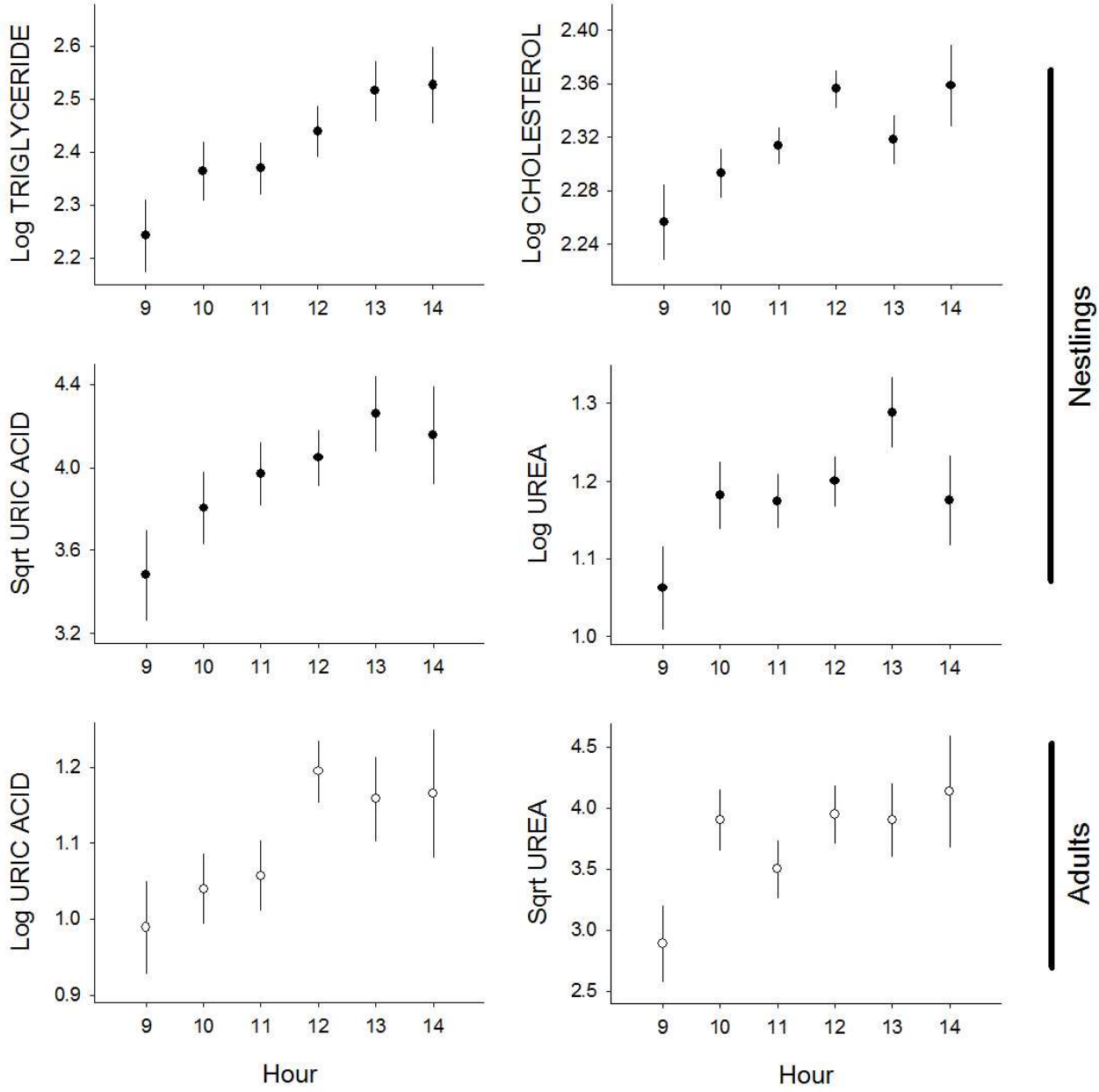


Fig. 2 Relationship between Triglyceride plasma levels and body mass (both variables standardized by regression of the other variables in the models in Table 2, i.e. leverage plots) for nestlings and adults. Dashed lines indicate 95 % confidence bands of simple linear regressions.

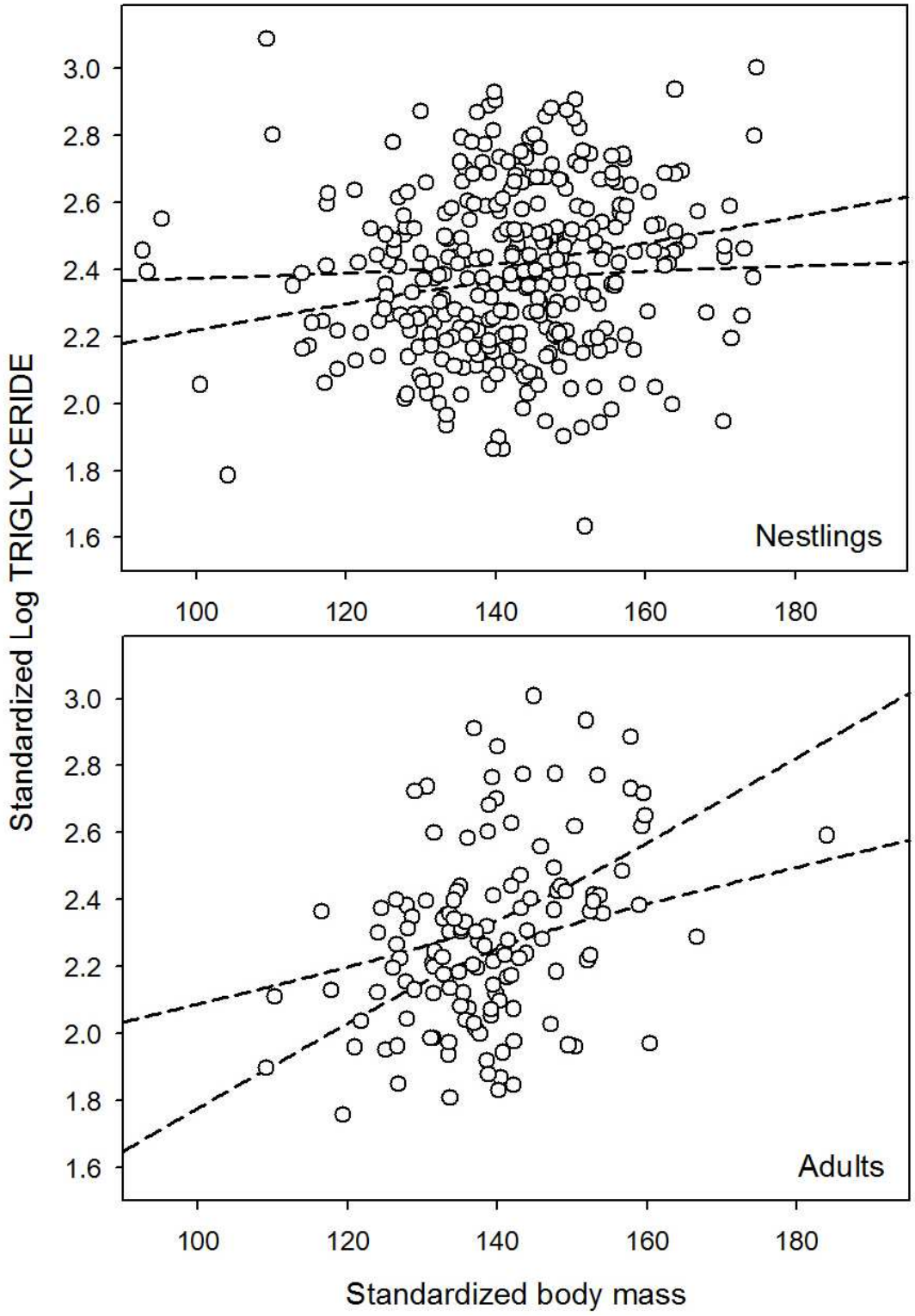


Fig. 3 Relationship between cholesterol (A) and triglycerides (B) levels of nestlings and number of nestlings. Least square means (± SE), from models including the significant terms from Table 2 and number of nestlings as an ordinal variable, are shown.

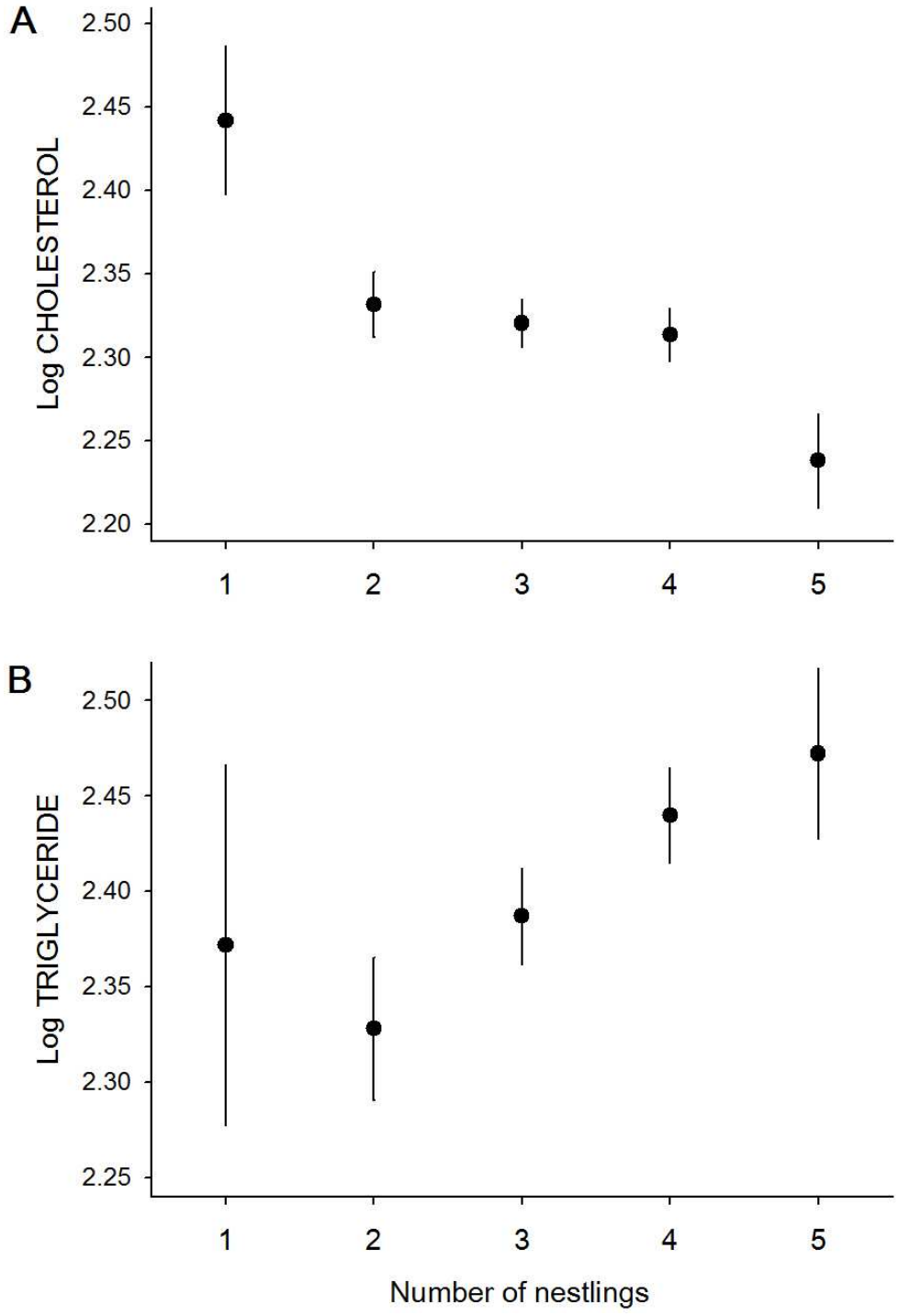
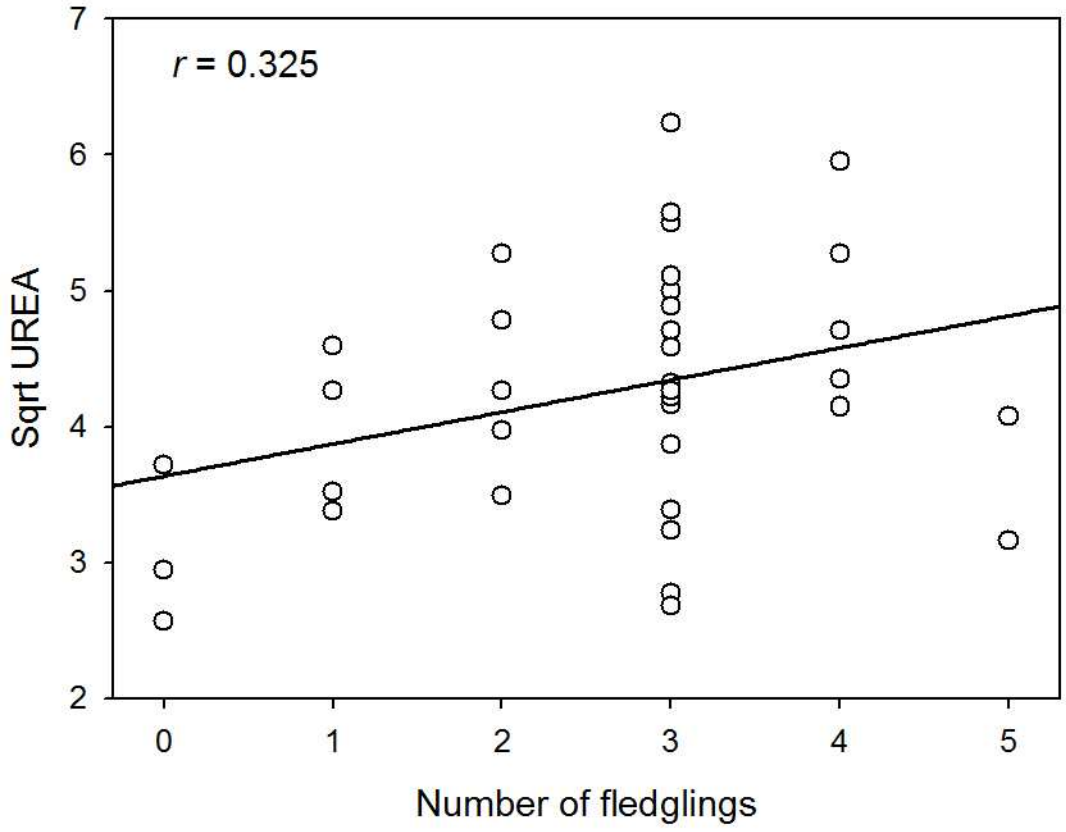


Fig. 4 Relationship between urea levels of adult lesser kestrels captured after June 10th and breeding success (measured as number of fledglings).



General discussion

Lesser kestrel is one of the best studied bird species in the world, considering than a majority of studies have been conducted on free-ranging populations. More than two hundred papers in international scientific journals and ten PhD thesis defended in several countries, as well as a huge number of articles in popular magazines have been published mainly reporting aspects of its breeding and foraging ecology, conservation status or genetics. This PhD attempts to fill a gap of knowledge on the migration ecology and migratory connectivity of the lesser kestrel. Furthermore, it explores for first time the general nutritional condition through plasma biochemical metabolites in the breeding grounds before migration.

Migration and wintering areas

Recent advances in telemetry and tracking have revolutionized the study of bird migration (reviewed in Robinson *et al.* 2010). The miniaturization of devices is the most significant aspect because it has let to tag a wide group of small birds. The miniaturized geolocators are currently the lightest devices (as light as 1 gram) which let us to track small passerine birds (Stutchbury *et al.* 2009). Although geolocators have been widely used in marine birds, their use in terrestrial species had been quite limited. Our study is the first one applying this technology in raptors. Eventhough several devices failed to provide information, the results were far-reaching. On the positive side, geolocators provided in one year of study more information than the ringing of thousands lesser kestrels during the last 30 years. We demonstrated conclusively that Spanish birds wintered in West Africa. On the negative side, we failed to determine the migration routes because of several factors: First, migration overlaps temporally with the equinoxes (when day and night time are approximately equal at all latitudes). At times near equinox, geolocators are not able to report accurate latitudinal estimates (Hill 1994). Second, only two locations are provided by day (Hill 1994) and given the apparent short time needed by kestrels to migrate (according to longitude

data 5 ± 1 and 24.3 ± 10 days for post- and pre-nuptial migrations, respectively), a small number of fixes are available, hindering us to use these data. Finally, we failed to get useful data from nine of the 13 retrieved geolocators. Due to the lack of previous studies, we decided to tag birds using backpack harnesses and PVC rings (typically used in marine birds). We found that geolocators mounted in backpack harnesses worked better than leg mounted ones. Thus, leg mounted geolocators showed damaged caused by bites, scratches and ingrained dirt. However, Catry *et al.* (2010) reported pre-nuptial migration routes based on geolocators leg mounted. According to this study, kestrels showed inter individual variations in migration routes, some birds flying hundreds of kilometers over the sea. Additionally, the estimated duration of migrations was similar to our data for the post-nuptial migration (4.8 ± 1.1 and 4.1 ± 0.3 days for post- and pre-nuptial migrations; Catry *et al.* 2010).

In chapter three, we used a non-invasive approach that relies on major histocompatibility complex (MHC) polymorphism to infer the breeding origin of individuals wintering in its most important wintering quarters in the Sub-Saharan Africa (Senegal and South Africa; LPO 2010, MKP 2010). To our knowledge, MHC genes have never been used for this purpose in birds, but in salmons is a relatively frequently technique used with other genetic markers to take appropriate decisions on the salmon fisheries management (Beacham *et al.* 2001, 2004). MHC genes of other migratory birds could be more structured than other nuclear markers (e.g. microsatellites), and thus letting us to use them migratory connectivity studies. For example, Great Snipe *Gallinago media* populations from Scandinavia and mainland Europe are highly structured populations (Ekblom *et al.* 2007). We found private alleles from western European breeding populations in the Senegalese roost, indicating a strong connectivity between these two areas. On the other hand, birds wintering in South Africa were genetically differentiated with respect to western European breeding populations, and might therefore gather individuals from the eastern distribution range (note that Asia was not adequately sampled). Our results coming from South Africa are similar to those reported by Wink *et al.* (2004) using mitochondrial DNA.

Our results on migration and wintering ranges are crucial for the development of conservation measures for the species. In this sense, Mihoub *et al.* (2010) assumed that French lesser kestrels wintered in Sahel region, and they found that yearling survival probabilities were strongly correlated with rainfalls in the Sahel, suggesting a high dependence of juvenile upon the wintering conditions.

Technical notes

In the second chapter, we assess the effect of gelocators on the breeding parameters of lesser kestrels. Except some collateral damages in individual birds (i.e., small wounds), no severe alterations in the breeding parameters were observed comparing tagged and untagged pairs. The 2007 and 2008 breeding seasons were exceptionally good for the reproduction of lesser kestrels (3.3 and 3.2 fledglings per successful nest, respectively). This may explain the lack of detrimental effects of geolocators.

Nestlings of tagged individuals had higher triglyceride and uric acid concentrations in their blood than control nestlings during the breeding season following tagging (2008). However, in posterior analyses including more nestlings from other years, this factor (presence/absence of tagged parents) did not reach significance (see chapter five). A high nestling mortality was observed in the 2009 breeding season, resulting in a lower breeding success (2.5 fledglings per successful nest) than in 2007 and 2008 breeding seasons. Therefore, the inclusion of nestlings with worse nutritional condition (those from 2009) in these more general analyses seems to explain the non-significance for this factor (see chapter five).

Working with MHC markers is not easy and several caveats must be highlighted. The characterization of MHC genes in non-model species, the co-amplification of different fragments of the multigene family during single PCR experiments and extensive levels of genetic polymorphism require a considerable set-up before using these markers (reviewed by Babik 2010). However, important advances for the amplification and genotyping of MHC genes in non-model species have been developed in the last years (Babik 2010). In chapter four, we test the accuracy of PHASE algorithm implanted in DNAsp software (Librado & Rozas 2009) to infer the gametic phase of PCR amplicons of class I and II MHC genes. Our results suggest that a minimum allele to individual ratio (1:2) to yield 100% accuracy for both MHC loci. This procedure let us to avoid the traditional and laborious cloning methods, saving money and time in the MHC genotyping.

Nutritional condition

As a consequence of logistic problems (the obtaining of an adequate sample size just before migration -August-September- is difficult because birds do not come into their nest-cavities, where capture is relatively easy), our study was conducted during the breeding period. As

far as we know, nutritional condition of lesser kestrels has not been previously evaluated using plasma biochemical markers, and therefore, ours is a novel study on the species biology, as well as on biochemistry and physiology of raptors. More robust statistical analyses than most studies that report blood metabolites in birds, especially raptors, are used in the present study. Furthermore, the relative large samples sizes let us to test the effect of multiple factors.

We found that nearly all ranges of plasma metabolites (triglycerides, cholesterol, uric acid and urea) for nestlings and adults overlapped with those described for other free-living raptors, suggesting that condition was similar to other falconiforms. However, we consistently detected higher mean values of triglycerides than in other raptors. These differences might be explained by the higher feeding frequency of lesser kestrels in comparison with the majority of studied species.

Several factors (year, colony, sampling time, body mass, laying date and capture date) influenced at least one plasma biochemical parameter. Sampling time was the most influential factor as it reached significance for nearly all parameters. As a consequence of the increase of feeding rate in the morning, nestlings showed a postprandial increase in all biochemical parameters analyzed. Uric acid and urea levels of adults also increased during the morning possibly because the increase of workload of reproduction duties. Despite sexual dimorphism of lesser kestrel and differences in the breeding behaviour, no sexual differences were detected for any biochemical parameter.

Future research

The present PhD tried to shed light on some previously unknown basic questions of life history traits of lesser kestrels. However, it also opens new questions. It is now clear that there are at least two main wintering grounds for lesser kestrels in Africa (Wink *et al.* 2004, Rodríguez *et al.* 2011). The western route is considerably shorter than the eastern one, raising interesting questions on energetic constraints and adaptations for medium or long-distance migration in birds travelling one or the other migration route. The development and miniaturization of satellite devices will let us know the duration of migration journeys, as well as determine the location of stopover sites. Relating sedentary/migrant behaviours and/or migration traits with breeding parameters could help to elucidate the trade offs of both strategies and their evolutionary consequences. Furthermore, GPS dataloggers could

be used on the wintering grounds to study habitat use and explain how lesser kestrels can avoid prey depletion in the surroundings of the crowded roosts (Bonal & Aparicio 2008).

A crucial question in conservation of this migratory species is to assess the migratory connectivity level between the different South African roosts and the breeding areas, i.e. the roost site fidelity. A strong connectivity (i.e. the breeding populations winter in single, mutually exclusive roosts) highlights the relevance for conservation of roost sites (Marra *et al.* 2006). This has been recently addressed using stable isotopes for the Amur falcon *Falco amurensis*, a species which share roosts with the lesser kestrels (Symes & Woodborne 2010). A similar methodology can be applied to lesser kestrels.

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Conclusions

1. Geolocators have failed to elucidate the migration routes, but they have provided invaluable information on the wintering areas of individuals with a known breeding origin. The lesser kestrels from the western European breeding range winter in West Africa. They show an apparent link with the deltas of Senegal and Saloum rivers.

2. Geolocators have no detrimental effects on the studied breeding parameters of lesser kestrels (clutch size, breeding success or laying date). However, nestlings with tagged parents showed higher levels of triglycerides and uric acid in blood than nestlings with control parents.

3. At least at a wide continental scale, Major Histocompatibility Complex class II genes are useful to determine the origin of lesser kestrels. Birds wintering in Senegal came from the Western breeding range (Europe), however birds wintering in South Africa came the Eastern breeding range. MHC resolution was similar to other typically used genetic markers on connectivity migration studies, as for example, mitochondrial DNA.

4. The use of PHASE algorithm to infer gametic phase is an accurate tool for the genotyping of MHC genes of the lesser kestrel. A critical influence of the allele to individual ratio on PHASE performance was detected. A minimum allele to individual ratio (1:2) yielded 100% accuracy for both MHC loci.

5. We consistently detected higher mean values of triglycerides in lesser kestrels than in other free-living raptors as a consequence of their small-sized prey. Lesser kestrels show higher feeding rates than other larger raptors, like eagles or vultures, keeping high values of triglycerides in blood.

6. Nestlings show a postprandial increase in all biochemical parameters analyzed because of the increase of feeding rate in the morning. Uric acid and urea levels of adults also increased during the morning possibly because the increase of workload.

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Tesis doctorales: TESEO

Resultado de la búsqueda

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- **Resumen:**

A pesar de que el cernícalo primilla Falco naumanni es una de las aves más estudiadas en el campo, poco se sabe de las áreas de invernada de las poblaciones reproductoras y de su condición nutricional. El principal objetivo de la presente tesis es aportar información básica sobre la conectividad migratoria de la especie entre las áreas de cría y de invernada. Nosotros usamos, por primera vez en rapaces, geolocalizadores para determinar las áreas de invernada de una población occidental de su rango de cría. Encontramos que los cernícalos invernaron en las inmediaciones de los ríos senegaleses Senegal y Saloum. Basándonos en datos de longitud, inferimos que la duración de la migración post-nupcial es 5 ± 1 días, mientras que la de la migración pre-nupcial es más larga y variable (24 ± 10 días). Los geolocalizadores no mostraron efectos perjudiciales en los adultos marcados (excepto un arnés mal fijado). La tasa de retorno fue similar a la observada en otros años y colonias (15 retornaron en 2008 de los 20 marcados en 2007); y el peso de individuos marcados no difirió de los no-marcados en el momento de la recaptura. Los parámetros de cría (tamaño de puesta y número de pollos volanderos) no fueron afectados por los geolocalizadores. Sin embargo, una mayor mortalidad de pollos de las parejas marcadas fue observada en 2008, así como un aumento en el nivel de triglicéridos y ácido úrico en el

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