COSTS AND BENEFITS OF EARLY REPRODUCTION: 
HAEMOPROTEUS PREVALENCE AND REPRODUCTIVE SUCCESS OF INFECTED MALE PIED FLYCATCHERS IN A MONTANE HABITAT IN CENTRAL SPAIN

COSTES Y BENEFICIOS DE FECHAS DE PUESTA TEMPRANAS: PREVALENCIA DE HAEMOPROTEUS Y ÉXITO REPRODUCTOR DE MACHOS INFECTADOS DE PAPAMOSCAS CERROJILLO EN UN HÁBITAT MONTANO EN EL CENTRO DE ESPAÑA

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SUMMARY.—Laying date is a key factor in avian reproductive ecology. Benefits of early breeding are important in terms of reproductive output. Costs are mainly associated to weather adversities at early stages of the breeding season. As males arrive earlier than females, they may face these weather adversities more frequently and therefore suffer higher costs at early stages of the season. We studied the potential association between the prevalence of infection by Haemoproteus, condition and age with the breeding phenology in male pied flycatchers Ficedula hypoleuca in a montane population in central Spain. Early males benefited from a greater reproductive success, but they may have paid costs in terms of a more marked seasonal relapse from Haemoproteus infections.

RESUMEN.—La fecha de puesta es clave en la ecología de la reproducción de las aves. Los beneficios de una reproducción temprana son importantes en términos de éxito reproductor. Los costes se asocian principalmente a las adversidades meteorológicas en fases tempranas de la temporada de cría. El hecho de que los machos generalmente lleguen antes que las hembras, hace que estos coincidan más frecuentemente con adversidades meteorológicas y por tanto sufran en mayor medida los costes de las fases tempranas de la temporada. Se explora la posible asociación entre la infección por Haemoproteus, condición y edad con fenología reproductiva en machos de papamoscas cerrojillo Ficedula hypoleuca en una población montana en España central. Los machos tempranos se beneficiaron en términos de un mayor éxito reproductor, pero sufrieron los costes de una más acusada recrudescencia estacional de las infecciones de Haemoproteus.

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Timing optimization at breeding is a major determinant of life-history evolution of migratory birds (Møller, 1994). The laying date is a good indicator of avian breeding phenology and may be conditioned by a wide range of factors including arrival dates to the breeding grounds, which may be dependent on individual condition, age, sex (Potti and Montalvo, 1991; Møller, 1994; Kokko, 1999; Morbey and Ydenberg, 2001; Smith and Moore, 2005), migratory conditions (Potti and Montalvo, 1991; Both et al., 2006), and, as it has been lately suggested, on climate change (Both et al., 2004; Møller, 2008).

There is clear evidence of seasonal declines in breeding success in birds from temperate areas (Siikamaki, 1998; Verhulst and Nilsson, 2008). Early breeding males ensure the acquisition of a territory, and are likely to occupy the best ones (Potti and Montalvo, 1991; Lundberg and Alatalo, 1992; Kokko, 1999; Smith and Moore, 2005). Additionally, early breeders may match seasonal peaks of food availability with the stage of parental care, which may result in higher offspring recruitment into the breeding population (Møller, 1994, Sanz et al., 2003; Smith and Moore, 2005). However, early breeding males may also suffer costs. Poor environmental conditions at early stages of the breeding season may have dramatic consequences on a large number of individuals (Potti and Montalvo, 1991; Møller, 1994; Brown and Brown, 2000; Newton, 2007). Resource limitations due to harsh weather conditions, added to the high physiological demands of migration and reproduction, could compromise individual condition (Levins, 1968; Gustaffson et al., 1994; Sheldon and Verhulst, 1996; Stjernman et al., 2008). In this stressful scenario, pathogenic agents such as viruses, bacteria and protozoa, may find a window in the immunological system of their hosts thereby producing spring infection relapses (tendency of latent infections to recur and become more acute during the breeding season, as a result of the trade-off between reproduction and immunity) (Nelson et al., 2002).

The genus *Haemoproteus* refers to a large number of species of intracellular parasitic protozoa (Haemosporida, Haemoproteidae) that infect red blood cells of birds all over the world (Valkiūnas, 1997). Parasites of genus *Haemoproteus* play an important role in avian evolutionary ecology. The pathogenicity may vary among different species of *Haemoproteus*. Donovan et al. (2008) reported evidences of severe disease and mortality caused by a certain type of *Haemoproteus* in 6 species of passerines. In other cases, *Haemoproteus* infection may not necessarily result in the death of the host, but may produce sublethal effects on physiology (Garvin et al., 2003b), or reduce reproductive success (Merino et al., 2000; Marzl et al., 2005). Additionally, *Haemoproteus* could induce a delayed arrival to the breeding grounds, which indirectly affects reproductive success (Møller et al., 2004). Sporozoites, the infective stage of *Haemoproteus*, are transmitted by haematophagus insect vectors which are broadly distributed (Valkiūnas, 2005). As a result, transmission of *Haemoproteus* parasites to Palaearctic birds may not be restricted to the breeding areas, as it may occur in the wintering grounds or even at the stopover sites during migration (Waldenström et al., 2002).

The pied flycatcher *Ficedula hypoleuca* is a small (12 - 13 g) trans-Saharan migratory hole-nesting passerine of European woodlands. It is a summer visitor that readily accepts artificial nest-boxes and is consequently very much used as a model species in studies of avian reproductive ecology (Lundberg and Alatalo, 1992; Moreno et al., 2005). Males arrive on average one week earlier than females and actively compete with other males for the best breeding sites in order to attract mates (Potti and Montalvo, 1991). Protandry, the earlier arrival date at the breeding grounds by males, is a common pattern in many animal taxa (Morbey and Ydenberg, 2001). Both, physiological (for example, hormones) and ecological (for example, competition for breeding territories) factors may explain a higher prevalence
of parasite infections in males (see Zuk and McKean, 1996 for a review). The physiological costs associated with egg production and incubation may act as strong evolutionary forces selecting females to avoid an early arrival to the breeding grounds (Nilsson, 1994). Pied flycatcher males competing for breeding territories show high levels of testosterone (Silverin, 1998), which has been suggested to enhance oxidative stress and reduce immunocompetence (Alonso-Álvarez et al., 2009). Most available data for birds support the “Sexual Selection” or “Vulnerable Male” Hypothesis, according to which males generally have higher probabilities to be parasitized than females (e.g. Moreno et al., 2001; see Hasselquist, 2007 for a review). Considering this evidence, we were motivated to specifically focus on males during the early stages of the breeding season.

The present study aims to investigate the potential association between breeding phenology (laying date) and infection by *Haemoproteus* in male pied flycatchers of a montane population breeding in central Spain. Male age and body mass were assessed, as they are known to influence parasite prevalence in small passerines (Weatherhead and Bennett, 1991; Sanz et al., 2001a; Valkiu纳斯, 2005). We predicted that early breeders may pay a price in terms of a higher susceptibility to *Haemoproteus* infection probably due to harsh weather conditions. Alternatively, early breeders are individuals in better condition and show a lower prevalence of *Haemoproteus* infection. Moreover, differences in reproductive success were also studied in relation to breeding phenology, phenotype (age and body mass) and infection by haemoproteids. We may predict that non-infected males or those in the best body condition and more experienced may show an earlier breeding phenology and therefore a greater reproductive success.

It should be noted that during this breeding season the population was subjected to a food supplementation experiment. From early stages of nest construction until one day after laying the last egg of the clutch, experimental breeding pairs were provided with live mealworms in a small container fixed under the entrance of the nest-box; in control pairs, the containers were empty. The outcomes of this experiment have been previously published with regard to egg mass and egg colouration and to effects on nestlings (see Moreno et al., 2006a and b; Moreno et al., 2008 for details). The experiment had no interference with the present study (see results).

This study was conducted during the spring of 2005 on a population of pied flycatchers breeding in artificial nest boxes in a deciduous forest of Pyrenean oak, *Quercus pyrenaica*, at 1,200 m above the sea level in the vicinity of La Granja, central Spain (40º 54´N, 04º 01´W) (see Moreno et al., 2005 for more details about the study population and area).

Nest boxes were visited daily. A total of 66 males were trapped when their chicks reached the age of 11 days. Age and body mass of each male were determined. For some males the exact age was known, as they were ringed in the study area as nestlings or had the plumage of their first year when captured (31 of 66 males were exactly aged). For the rest of males we assumed that they were first captured in the study area as breeders with a minimum age estimate of two years. Birds were weighed with a Pesola (Baar, Switzerland) spring balance to the nearest 0.1g.

Parasite prevalence (infected vs. uninfected) is a reliable measure of the status of infection. In this study, males were divided into two groups, those infected by *Haemoproteus* spp. and those which were uninfected. To assess the status of infection by haemoproteids a blood sample was obtained from the brachial vein of the birds. A drop of blood was immediately smeared on individually marked microscope slides and air-dried. Blood smears were later fixed in absolute ethanol and stained with Giemsa (1/10 v/v) for 45 min. One half longitudinal section of each
smear was scanned under oil immersion at 1,000x in search of *Haemoproteus* parasites (Merino and Potti, 1995; Merino et al., 1997). When an infected erythrocyte by *Haemoproteus* sp. was detected, the individual was then assigned to the infected group.

Statistical analyses were conducted using *Statistica 6.0* (StatSoft 2001). Generalized linear models (GLZ) with binomial distribution were developed to study the possible association between the prevalence of *Haemoproteus* (dependent categorical variable) with laying date, male age and body mass. Male age and body mass were considered as continuous predictors and laying date as a categorical factor. A bimodal distribution of frequencies observed for laying date allowed us to define two categories: early (mean = 41.37, range: 36 - 45) and late layers (53.24, 49 - 60) (laying date 1 = 1st April). Between days 45 - 49, new clutch initiation was only detected in one nest (day 48), although no blood sample could be obtained from the male of this nest and thus it was not included in the analyses. When exploring the distribution of frequencies of laying date for all the nests occupied during the breeding season (including 18 nests for which the blood sample was not obtained; n = 84), the bimodality of laying date was still consistent. Thus, the bimodality of laying date observed for the 66 nests included in this study was the pattern in the study population during the breeding season and not an artefact of subsampling.

A GLZ with ordinal multinomial distribution was performed to study the relationships between reproductive success (number of fledglings), laying date, infection by *Haemoproteus*, male age and body mass. Final models were obtained by a backward deletion procedure. We first obtained the full model, including all possible predictors; variables were then sequentially removed when the variance explained did not significantly improve the model ($\alpha = 0.05$).

The food supplementation experiment had no significant effects, neither on the prevalence of *Haemoproteus* in males (Chi-Squared Test, $X^2_{1} = 0.17, N = 66, P = 0.68$) nor on the number of fledged chicks (Wald Test, Wald Stat. $= 0.16, N = 66, P = 0.68$). In addition, to prevent any other confounding effects of the experiment on the current study, we further analyzed some potentially important relationships: (i) supplemented males did not significantly differ in body mass from control males at the end of the nestling period (Anova, $F_{1,65} = 0.46, P = 0.50$); however, the effects of food supplementation on body mass could not be ascertained directly due to the high risk of male desertion if captured at early stages of breeding; (ii) control and supplemented nests did not differ in the duration of the experiment, laying date, or clutch size (Moreno et al., 2006a); (3) reproductive success ($n$º fledglings / $n$º eggs) was not significantly affected by food supplementation (Mann-Whitney Test, $Z = -0.25, N = 66, P = 0.80$).

Twelve out of 66 males were infected by *Haemoproteus* spp. A significant relationship was found between the prevalence of *Haemoproteus* parasites and laying date, early breeding males being more likely to be infected (Wald Test, Wald Stat. $= 5.23, N = 66, P = 0.02$; fig.1). Male age and body mass were not associated with *Haemoproteus* prevalence (Wald Test, Wald Stat. $= 0.66, N = 65, P = 0.42$; Wald Stat. $= 0.21, N = 65, P = 0.65$, respectively). Likewise, laying date was the best explanatory variable in the final model of the number of fledglings, early breeders showing greater reproductive success (Wald Test, Wald Stat. $= 4.25, N = 66, P = 0.04$). Male age and body mass had no effect on reproductive success (Wald Test, Wald Stat. $= 2.02, N = 65, P = 0.15$; Wald Stat. $= 0.20, N = 65, P = 0.65$, respectively). Finally, the associations of laying date with male age and body mass were not significant (Anova, $F_{1,65} = 0.22, P = 0.64$; $F_{1,65} = 1.56, P = 0.22$, respectively).

Although the correlative nature of this study does not allow determining causal relationships, the significant trends obtained confirm...
the key importance of laying date in avian life history evolution (Møller, 1994).

We found that early breeding males produced more fledglings. The interpretation of this relationship seems straightforward, coinciding with the broad evidence for seasonal declines in breeding success in temperate areas (Siikamaki, 1998; Verhulst and Nilsson, 2008). This could be due to the fact that only the most experienced individuals or those in good condition might be able to arrive early to the breeding grounds and start reproducing early (Potti and Montalvo, 1991; Møller, 1994; Kokko, 1999; Smith and Moore, 2005). However, no association was found between laying date and male age or body mass. Intensified competition due, for instance, to huge differences in quality between territories, may deviate arrival schedules from the expected general trend (Kokko, 1999). Theoretically, if marginal costs of advancing arrival are larger for individuals in low condition, early males should be those in high body condition. This would favour that high quality individuals occupy prime territories (Møller, 1994). However less experienced males or those in low condition may arrive early when there are large payoffs for them, e.g. if best territories are much better than the rest (Kokko, 1991). It is unclear if this may happen in our population, as early breeding in a montane habitat may entail costs due to harsh weather conditions. We may also speculate that intensified competition for territories already in the winter quarters may have resulted in a cascading arrival, as suggested by Kokko (1999) for the com-

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**Fig. 1.—**Number of male pied flycatchers uninfected and infected by *Haemoproteus* in relation to laying date categories (early (mean = 41.37, range: 36 - 45) and late layers (53.24, 49 - 60) (laying date 1 = 1st April)). Numbers on top of bars represent sample size. Within the early males and late males categories, the prevalence of *Haemoproteus* was of 50 % and 10.53 % respectively.

[Número de machos de papamoscas cerrojillo no infectados e infectos por Haemoproteus en relación a las categorías de fecha de puesta (fecha de puesta temprana (media = 41.37, rango: 36 - 45) y tardía (53,24, 49 - 60). Los valores mostrados sobre las barras indican el tamaño de muestra. Dentro de la categoría de machos tempranos y tardíos, la prevalencia de Haemoproteus fue del 50 % y 10,53 % respectivamente.]
petition between males in the breeding grounds. Also, it should be noted that males were weighed when chicks reached 11 days. The time elapsed since laying date to the date when males were weighed may have obscured the potential relationship between laying date and male condition.

Furthermore, differences in male breeding phenology were associated with differences in *Haemoproteus* prevalence, early breeding males being more likely to show infection by *Haemoproteus* sp. Different lineages of *Haemoproteus* may be transmitted in both, wintering areas in Africa or breeding areas in Europe (Waldenström et al., 2002). Migratory birds parasitized while wintering in Africa may suffer the costs of infection during the pre-breeding migration (Waldenström et al., 2002; Valkiūnas, 2005), for instance, in terms of a delayed arrival to the breeding areas (Møller et al., 2004). Therefore, our results may seem to contradict the general idea that early breeders are those in better health. Although *Haemoproteus* infection could be an important causal factor explaining delayed arrival (Møller et al., 2004), in our study infections would rather be a consequence of early arrival and breeding. A higher prevalence of infection observed in early breeding males may have resulted from experiencing different costs with respect to late breeding males. For instance, early breeding males may have suffered a loss of physiological condition as a consequence of sudden early spring cold spells, which are common in these montane habitats (Potti and Montalvo, 1991; Sanz, 1994). This added to the general costs of migration and reproduction may cause a loss of physiological condition in early breeding males (Sheldon and Verhulst, 1996; Gustafsson et al., 1994; Stjernman et al., 2008) and consequently, a higher susceptibility to infection or a more marked parasite seasonal relapse of previous infections. Correlative and experimental evidence support recurrences of *Haemoproteus* infections during the breeding season (Garvin et al., 2003a; Valkiūnas et al., 2004; but see Hasselquist et al., 2007). The prevalence of infection must be carefully interpreted according to the blood sampling date in relation to laying date (Weatherhead and Bennet, 1991; Garvin et al., 2003a). It should be noted that blood sampling in the current study was performed when chicks reached 11 days of age, while, for example, Møller et al. (2004) did it soon after the arrival of males on the breeding grounds. Thus, an alternative explanation is that early breeders performed a higher reproductive investment throughout the breeding season, which resulted in a higher susceptibility to haemoproteids, as reported by Norris et al. (1994) for other Haematozoan parasites.

No association was found between male body mass and age with *Haemoproteus* prevalence. Recent experimental and correlative studies have repeatedly shown detrimental effects of this parasite in individual body mass in different bird species and life stages (Merino et al., 2000; Garvin et al., 2006; Valkiūnas et al., 2006). Following this line of evidence, Dawson and Bortolotti (2000) found that during incubation male American kestrels infected by *Haemoproteus* had significantly lower body condition than uninfected males; however, this association was only detected in one of the two years of the study and only during the incubation period. Furthermore, infected females were in poorer body condition than those without parasites during incubation but not prior to egg laying. On the contrary, other studies failed to detect effects of *Haemoproteus* on body mass in passerines (Bennet et al., 1988). Dawson and Bortolotti (2000) argued that variation among factors such as the pathogenicity of the parasite, for example, could explain positive, negative or even the absence of association between infection and body condition. Other possibilities are that the infection by *Haemoproteus* had detrimental effects in other unmeasured aspects of physiological condition, such as immunocompetence, or that the negative effects are only
detectable in the long term. For instance, infected birds could show lower return rates, as found by Dawson and Bortolotti (2000) for the American kestrel.

There is a very patchy and sometimes even contradictory literature about haemosporidians prevalence in different bird age classes (Valkiūnas, 2005). Many studies have coincided in detecting an increase of *Haemoproteus* prevalence with age-classes in passerine birds (Weatherhead and Bennet, 1991; Allander and Bennett, 1994; Sanz et al., 2001a; Deviche et al., 2001). In general, authors agreed that this trend may result from differences in the exposure to vectors between young and older birds. Sol et al. (2000) experimentally demonstrated that vector abundance is the main factor influencing the spatial variation in the prevalence of *Haemoproteus* in pigeons. In addition, local weather conditions may be important determinants of haematophagus insect vectors abundances (Martínez de la Puente et al., 2009). However, the absence of association between *Haemoproteus* prevalence and different age-classes found in our study has repeatedly been reported in different species of passerines (e.g. Ratti et al., 1993; Gibb et al., 2005; Garvin et al., 2006; Hasselquist et al., 2007; Hartup et al., 2008). Since there was no difference in breeding date according to male age, early breeders of different age-classes might have been exposed to the same vectors and weather conditions, which may have led to a lack of association between *Haemoproteus* prevalence and age.

Several studies have reported costs of infection by *Haemoproteus* spp. in reproductive success in pied flycatchers (Siikamäki et al., 1997; Sanz et al., 2001b; Morales et al., 2006). Moreover, direct experimental evidence that *Haemoproteus* infections have detrimental effects on reproductive success in other wild passerines has also been reported (e.g. Merino et al., 2000; Marzial et al., 2005). Despite these facts, we were not able to detect a significant association between *Haemoproteus* infection and the number of fledglings in the present study. It seems that the benefits of breeding early in terms of reproductive success outweighed the cost of being parasitized. Infection by *Haemoproteus* in early breeders, which could have long-term effects on reproductive performance, thus reducing the probability of survival in infected individuals (Dawson and Bortolotti, 2000; Sol et al., 2003), is a possibility which cannot be excluded. A real understanding of avian ecology and evolution will require a long-term approach to the study of parasite-host interactions (Loye and Zuk, 1991; Fitze et al., 2004; Valkiūnas, 2005; Thomas et al., 2005). However, this may also be due to an adjustment of reproductive investment to physiological conditions for each individual (Gustaffson et al., 1994). Further experimental manipulation of parasite loads is necessary to study the effects of *Haemoproteus* infection on reproduction.

In conclusion, our results add to previous evidence that early breeding phenology maximizes reproductive success. However, early breeders seem to suffer costs in terms of increased *Haemoproteus* infection. This could be due to adverse weather conditions at the onset of reproduction, especially in montane habitats, which may favour seasonal parasite relapses.

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