Ibis (2009), **151**, 559–567

Physiological response to stress in fledgling Lesser Kestrels *Falco naumanni*: the role of physical condition, sex and individual genetic diversity

JOAQUÍN ORTEGO,[1]* LAURA ZAPERO,[2] GUSTAU CALABUIG,[2] PEDRO J. CORDERO[2] & JOSÉ MIGUEL APARICIO[2]

[1]*Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), C/ José Gutiérrez Abascal 2, E-28006 Madrid, Spain*

[2]*Grupo de Investigación de la Biodiversidad Genética y Cultural, Instituto de Investigación en Recursos Cinegéticos - IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, E-13005 Ciudad Real, Spain*

Exposure to chronic stress early on during development has important deleterious consequences later in life, reducing important components of individual fitness such as survival and future reproduction. In this study, we evaluate the factors associated with physiological response to stress in fledgling Lesser Kestrels *Falco naumanni*, paying particular attention to the potential role of individual genetic diversity. For this purpose, we used heterophil/lymphocyte ratios (H/L ratio) as a haematological stress indicator and typed the analysed individuals at 11 highly polymorphic microsatellite loci, which allowed us to estimate their genetic diversity. We found that the H/L ratio decreases with fledgling physical condition, suggesting that this parameter is a good indicator of nutritionally based physiological stress. Physiological response to stress was higher in males than in females and this effect was independent of physical condition, suggesting that the observed pattern is due to inherent sexual differences in the factors influencing H/L ratios. Finally, the H/L ratio was positively associated with the genetic diversity of offspring. Previous experimental studies have found that individuals with higher genetic diversity show increased levels of circulating glucocorticoids, which in turn are directly responsible for increasing H/L ratios. On this basis, we suggest that a positive effect of genetic diversity on corticosterone levels may explain the observed association between H/L ratios and individual heterozygosity. Overall, this study highlights the utility of leucocyte profiles to study stress in wild bird populations and poses an interesting question about the effects of individual genetic diversity on haematological response to stress.

Keywords: chronic stress, corticosterone, glucocorticoids, heterozygosity, leucocyte, microsatellites.

Exposure to long-term stress has deleterious consequences, reducing important components of individual fitness such as survival and future reproduction (Merino *et al.* 2006). Especially important are stresses that occur in early development as they can ultimately have severe consequences later in life (Lobato *et al.* 2005, Blas *et al.* 2007). Stress during early-life stages frequently occurs in species with parental care due to the constraints on the quantity of food that parents can supply relative to the energy demand of their offspring (Suorsa *et al.* 2004, Arriero *et al.* 2008). Apart from food shortage, a number of other extrinsic factors such as parasitism, sibling aggression, predator presence, temperature and human disturbance have been identified as important stressors in animal populations (e.g. Merino *et al.* 2002, Davis *et al.* 2004, Suorsa *et al.* 2004, Lobato *et al.* 2005, Arriero *et al.* 2008). Intrinsic characteristics of individuals can also influence their physiological response to stress, including sex, age, reproductive status and individual genetic diversity (Kristensen *et al.* 2002, Merino *et al.* 2002,

*Corresponding author.
Email: joaquin.ortego@mncn.csic.es

Pedersen *et al.* 2005, Davis & Maerz 2008). Reduced genetic diversity due to inbreeding, founder/bottleneck effects and/or small population size, may affect the physiological response to stress if genes that affect stress tolerance are also affected by reduced genome-wide heterozygosity, or if the expression of partly deleterious recessive alleles disturbs cellular homeostasis and protein regulation (Kristensen *et al.* 2002, Pedersen *et al.* 2005). Further, there is some evidence that reduced genetic diversity decreases the competitive ability and aggressiveness of individuals and this can ultimately result in higher levels of stress (Meagher *et al.* 2000, Tiira *et al.* 2003, 2006). However, the possible effects of individual genetic diversity on physiological response to stress have been hitherto rarely investigated and the scarce studies published on the topic are based on laboratory species (Kristensen *et al.* 2002, Pedersen *et al.* 2005).

Different parameters have been used to estimate stress in free-living vertebrates (e.g. Vleck *et al.* 2000, Suorsa *et al.* 2003, 2004, Arriero *et al.* 2008, Davis *et al.* 2008). One of the most popular methods of physiological stress assessment is the ratio of heterophils to lymphocytes in peripheral blood (Davis *et al.* 2008). This parameter has been suggested as a good indicator of physiological response to stress for several reasons. First, its levels are known to rise in response to most stressors, including infection, disease, food or water deprivation, extreme temperatures, or exposure to novel social conditions (Gross & Siegel 1983, Maxwell & Robertson 1998, Suorsa *et al.* 2004). Secondly, the heterophil/lymphocyte ratio is a good indicator of chronic stress and it is less variable and longer lasting than other physiological parameters such as plasma levels of corticosteroids (Davis *et al.* 2008). Thirdly, the heterophil/lymphocyte ratio correlates with other indexes of physiological stress such as circulating levels of heat-shock proteins (Moreno *et al.* 2002) and is sensitive to increased plasma corticosterone levels (e.g. Morici *et al.* 1997, Davis *et al.* 2000, Kranendonk *et al.* 2005, reviewed in Davis *et al.* 2008). Finally, it is relatively inexpensive and easy to determine using the traditional white blood cell counts in blood smears, which can be obtained without major disturbance of the study populations (Vleck *et al.* 2000, Davis *et al.* 2008).

In this study, we analyse the factors associated with stress during development in fledgling Lesser Kestrels *Falco naumanni*, a colonial and hole-nesting bird of prey in which both parents contribute to incubation and parental care. We are particularly interested in studying the association between individual genetic diversity and physiological response to stress, which to the best of our knowledge has never been explored in any wild population (Davis *et al.* 2008). For this purpose, we used heterophil/lymphocyte ratios as a haematological stress indicator and typed the analysed individuals at 11 highly polymorphic microsatellite loci, which allowed us to estimate their genetic diversity.

METHODS

Study population and field procedures

The study was conducted in La Mancha, central Spain (600–800 m asl), in an area covering approximately 1000 km^2 (Ortego *et al.* 2007a). During the 2004 breeding season, we studied 22 Lesser Kestrel colonies clustered in two localities separated by 30 km: 'Villacañas' (39ª30′N, 3ª20′W; 16 colonies) and 'Consuegra' (39ª35′N, 3ª40′W; 6 colonies). Lesser Kestrels normally arrive at their breeding grounds from their winter quarters in Africa in mid-February or the beginning of March (Calabuig *et al.* 2008). Egg-laying lasts from the end of April to the first week of June (Aparicio & Bonal 2002). They are primarily monogamous and lay only one clutch per season (modal clutch size in our study population = 4 eggs; range: 1–6 eggs) with the exception of rare replacement clutches (*c.* 0.5%), which were excluded from this study. We located nest-sites before the onset of egg-laying and each potential nest was regularly examined to determine laying date. The laying date was defined as the date the first egg was laid (Aparicio & Bonal 2002). Adults were trapped with a noose carpet or by hand during incubation, measured and individually marked with metallic and coloured plastic rings. To age adult birds that were not ringed as nestlings we assumed that individuals captured for the first time were in their first year if they presented yearling plumage or in their second year if they presented adult plumage (e.g. Aparicio & Cordero 2001, Foerster *et al.* 2003). Young were marked at hatching with a waterproof felt-tip pen, and they were banded 5–7 days later (for more details on field methods, see Aparicio 1997). During this early visit to the nest we estimated the hatching order of the chicks according to the observed mass hierarchy within the brood, which

accurately reflects the hatching sequence (Aparicio 1997). Hatching order was encoded as first, middle or last hatched eggs, allowing us to compare hatching sequences among different clutch sizes (e.g. Martínez-Padilla *et al.* 2004).

We captured a total of 302 adult Lesser Kestrels (135 males and 167 females). Of these, 169 individuals were captured in the Villacañas subpopulation (75 males and 94 females) and 133 in the Consuegra subpopulation (60 males and 73 females). For the purpose of the present study we have focused on 77 nests (180 fledglings) where both parents were captured (43 nests with 120 fledglings in Villacañas subpopulation and 34 nests with 60 fledglings in Consuegra subpopulation).

We used pectoral thickness to estimate fledgling and adult body condition (Aparicio 1997, Aparicio & Cordero 2001). By 'condition', we mean the value of a quantitative phenotypic trait that has a strong influence on fitness (Aparicio & Cordero 2001). In Lesser Kestrels pectoral thickness is positively correlated with the probability of recruitment of young and therefore it may be a realistic measure of body condition in this species (Aparicio 1997, Aparicio & Cordero 2001). This trait has also been used in previous studies as a measure of body condition in several bird species (Bolton *et al.* 1991, Newton 1993), and has been considered a more reliable measure of condition than residuals of body mass on tarsus length (Gosler & Harper 2000). Moreover, it is easy to measure accurately on live birds by using a portable ultrasonic meter, in this case a Krautkrämer USM22F (accuracy 0.1 mm; Hürth, Germany), especially designed to measure animal tissues. An ultrasonic meter measures the distance between a sensor (positioned in the pectoral muscle surface) and a material discontinuity (in this case the sternum bone) using reflected ultrasound. Breast feathers were soaked with alcohol and then carefully parted to place the sensor on the skin surface at 7 mm from the sternum (Aparicio 1997). This technique has been described in detail and tested on Mute Swans *Cygnus olor* by Sears (1988). The pectoral thickness of all the chicks within a brood was measured when the oldest sibling was 30 days old. Nestlings were sexed at the age of 20–30 days by the colour of their tail feathers and upper tail coverts. This method was 100% reliable, as assessed by the accuracy of sex determination of offspring that were subsequently recaptured in the following breeding seasons displaying unequivocal sexual plumage dichromatism (Aparicio & Cordero 2001). Adult and nestling blood samples (100 μL) were obtained by venipuncture of the brachial vein and preserved in ~1.2 mL 96% ethanol at −20°C.

Leucocyte counts

A drop of blood from each chick within a brood was smeared on individually marked microscope slides when the oldest sibling was 30 days old (i.e. close to fledging). Each smear was rapidly air-dried, fixed with absolute ethanol and stained in the laboratory with Giemsa's solution (1/10) for 45 min. The proportion of different types of leucocytes was assessed on the basis of a count of 100 leucocytes under oil immersion at ×1000 magnification (Ortego & Espada 2007). Fields with similar densities of erythrocytes were scanned for all individuals (Lobato *et al.* 2005). We also recorded the number of fields scanned during the leucocyte count as well as the number of red blood cells per field. This allowed us to estimate leucocyte concentrations by calculating the number of leucocytes observed per 2000 erythrocytes (e.g. Horak *et al.* 1999, Ortego & Espada 2007).

Genotyping and genetic diversity estimates

We genotyped Lesser Kestrels across 11 highly polymorphic microsatellite markers: Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp86-2, Fp89 (Nesje *et al.* 2000), Fu1, Fu2 (J.H. Wetton unpubl. data), Fn1-11 and Fn2-14 (Ortego *et al.* 2007b, see Ortego *et al.* 2007c for microsatellite details). All individuals were genotyped at all 11 microsatellite markers. We used QIAamp DNA Blood Mini Kits (QIAGEN, Hilden, Germany) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μL reaction volumes containing 1× reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer; Ecogen, Madrid, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, HEX or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf, Hamburg, Germany) thermal-cycler. The PCR programme used was 9 min denaturing at 95°C followed by 30 cycles of: 30 s at 94°C, 45 s at the annealing temperature (Ortego *et al.* 2007b, 2007c)

and 45 s at 72°C, ending with a 5-min final elongation stage at 72°C. Amplification products were electrophoresed using an ABI 310 Genetic Analyser (Applied Biosystems, Foster city, CA, USA) and genotypes were scored using GeneScan 3.7 (Applied Biosystems). We used two metrics to estimate individual genetic diversity of both parents and offspring: (i) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous; (ii) heterozygosity by loci (*HL*), a microsatellite-derived measure that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability (Aparicio *et al.* 2006). *HL* is calculated as follows: $HL = (\Sigma E_h)/(\Sigma E_h + \Sigma E_j)$, where E_h and E_j are the expected heterozygosities of the loci that an individual bears in a homozygosis (h) or heterozygosis (j) state, respectively (Aparicio *et al.* 2006). It should be noted that *HL* shows higher values in homozygous individuals and thus this index is negatively associated with individual genetic diversity (Aparicio *et al.* 2006). H_O and *HL* were calculated using CERNICALIN, an EXCEL spreadsheet available on request.

Statistical analyses

We examined the factors influencing heterophil/lymphocyte ratio using a generalized linear mixed model (GLMM) implemented with the GLIMIX macro of SAS (SAS Institute 2004). GLMMs allow analyses of data where the response variable is determined by both random and fixed effects. Heterophil/lymphocyte ratio in fledgling Lesser Kestrels was analysed using a normal error structure and an identity link function. As explanatory variables we fitted into the model genetic terms (offspring and maternal and paternal genetic diversity) and all non-genetic parameters (covariates: fledgling pectoral thickness, hatching order, fledgling age, laying date, brood size, maternal and paternal pectoral thickness and age; fixed factor: fledgling sex). The identities of natal colony and subpopulation were included as random effects to control for the potential non-independence of physiological stress within colonies and localities, in the manner of a randomized complete block design to avoid pseudoreplication (Krackow & Tkadlec 2001). Given that siblings were not independent among themselves, we also included brood identity nested within colony identity (i.e. higher-level factor; for the rationale of the model, see Singer 1998) as random effect. Initially, the GLMM was constructed with all explanatory terms fitted, including first-order interactions and quadratic effects to account for potential nonlinear relationships. The final model was selected following a backward procedure, by progressively eliminating non-significant variables ($P < 0.05$). The significance of the remaining variables was tested again until no additional variable reached significance. The result is the minimal most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. Denominator df were computed using Satterthwaite's method. This method provides a numerical approximation of ddf, so the reported ddf are not necessarily whole numbers (SAS Institute 2004). Hypotheses were tested using *F*-statistics and all *P*-values refer to two-tailed tests.

RESULTS

The heterophil/lymphocyte ratio was higher in males than in females (Table 1; Fig. 1) and decreased with fledgling physical condition estimated by pectoral thickness (Table 1; Fig. 2a). The heterophil/lymphocyte ratio was negatively associated with fledgling heterozygosity by loci (*HL*; Table 1; Fig. 2b) and positively associated with uncorrected heterozygosity (H_O: $F_{1,172} = 5.51$, $P = 0.020$). This GLMM accounted for the 21.5% of the original deviance. None of the other parameters, quadratic terms or interactions between independent variables was significant ($P > 0.05$). The relationship between *HL* or H_O and the heterophil/lymphocyte ratio was also significant when each metric was included alone in the model (*HL*: $F_{1,172} = 4.32$, $P = 0.039$; H_O: $F_{1,173} = 4.97$, $P = 0.027$), so that the association observed was not due to interactions among independent variables. We also analysed whether heterozygosity correlates with pectoral thickness to explore possible indirect effects of genetic diversity on the heterophil/lymphocyte ratio. After controlling for random effects (colony identity, nest identity and subpopulation), we found no significant association between *HL* ($F_{1,167} = 0.01$, $P = 0.965$) or H_O ($F_{1,167} = 0.01$, $P = 0.907$) and fledgling pectoral thickness. Finally, we explored whether a particular locus significantly influenced the association between heterophil/lymphocyte ratio and individual genetic diversity. After removing any single

Table 1. GLMM for the heterophil/lymphocyte ratio in fledgling Lesser Kestrels (normal error and identity link function) in relation to multilocus genetic diversity of parents and offspring (*HL*) and non-genetic terms (covariates: fledgling pectoral thickness, hatching order, fledgling age, laying date, brood size, maternal and paternal pectoral thickness and age; fixed factor: fledgling sex). Parameter estimates ± se for the levels of fixed factor were calculated considering a reference value of zero for the 'female' level in the variable 'sex'.

	Estimate ± se	Test	*P*
Explanatory terms			
Intercept	1.236 ± 0.238		
Sex	−0.084 ± 0.034	$F_{1,159} = 6.15$	0.014
Fledgling pectoral thickness	−0.075 ± 0.025	$F_{1,128} = 9.19$	0.003
Heterozygosity by loci (*HL*)	−0.348 ± 0.138	$F_{1,175} = 6.33$	0.013
Rejected terms			
Fledgling age		$F_{1,161} = 0.02$	0.883
Hatching order		$F_{1,117} = 3.56$	0.062
Laying date		$F_{1,65.4} = 0.16$	0.689
Brood size		$F_{1,83.8} = 3.04$	0.085
Maternal *HL*		$F_{1,49.9} = 0.45$	0.507
Paternal *HL*		$F_{1,49.3} = 0.88$	0.351
Maternal pectoral thickness		$F_{1,50.3} = 0.24$	0.626
Paternal pectoral thickness		$F_{1,46.8} = 0.03$	0.863
Maternal age		$F_{1,49.3} = 1.04$	0.313
Paternal age		$F_{1,37.5} = 0.05$	0.824
Covariance parameter estimates			
Subpopulation	0	–	–
Colony identity	0.002 ± 0.003	$Z = 0.66$	0.255
Brood identity	0.012 ± 0.007	$Z = 1.89$	0.029

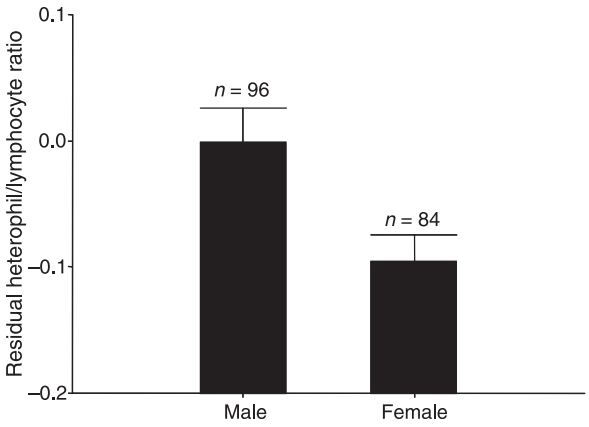

Figure 1. Heterophil/lymphocyte ratio (mean ± se) for male and female fledgling Lesser Kestrels. The heterophil/lymphocyte ratios are expressed as statistical residuals obtained after controlling for other influential variables (see Table 1).

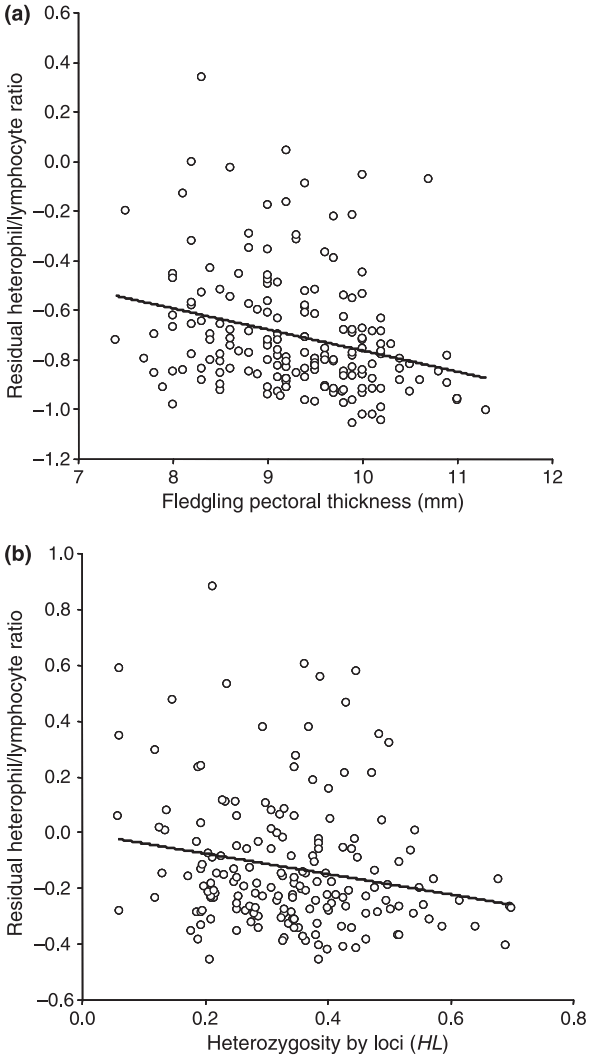

Figure 2. Relationship between heterophil/lymphocyte ratio and (a) fledgling pectoral thickness and (b) heterozygosity by loci (*HL*). The heterophil/lymphocyte ratios are expressed as statistical residuals obtained after controlling for other influential variables (see Table 1).

locus from the calculation of *HL* and H_O, the relationship between both measures of heterozygosity and heterophil/lymphocyte ratio always remained significant (Table 2). Of the 11 markers used, only one (Fp79-4) revealed an individual significant association with heterophil/lymphocyte ratio. However, after applying sequential Bonferroni correction for multiple tests no single locus effect remained significant.

Finally, we analysed whether the relationship between heterophil/lymphocyte ratio and physical condition, sex and individual genetic diversity was

Table 2. Tests for the effects of single locus and general heterozygosity (all loci, measured as *HL* and H_o excluding the locus being considered) on heterophil/lymphocyte ratio of fledgling Lesser Kestrels. Table shows *P*-values.

	HL		H_O	
Locus	Single locus	General	Single locus	General
Fp5	0.636	0.014	0.605	0.014
Fp13	0.786	0.013	0.763	0.013
Fp31	0.164	0.036	0.163	0.036
Fp46-1	0.138	0.031	0.136	0.035
Fp79-4	0.035	0.044	0.031	0.036
Fp86-2	0.557	0.016	0.546	0.017
Fp89	0.943	0.009	0.995	0.009
Fu1	0.522	0.017	0.553	0.017
Fu2	0.528	0.003	0.567	0.006
Fn1-11	0.129	0.037	0.120	0.033
Fn2-14	0.725	0.014	0.629	0.017

particularly mediated by a specific third association between these variables and lymphocyte or heterophil concentrations. We found that lymphocyte concentration was not associated with individual genetic diversity (*HL*: $F_{1,171} = 2.80$, $P = 0.096$; H_O: $F_{1,171} = 3.04$, $P = 0.083$), sex ($F_{1,170} = 0.02$, $P = 0.875$) or pectoral thickness ($F_{1,112} = 1.77$, $P = 0.186$). On the other hand, heterophil concentration was negatively associated with fledgling pectoral thickness ($F_{1,125} = 10.50$, $P = 0.002$) and was higher in males ($F_{1,165} = 5.02$, $P = 0.026$). However, heterophil concentration was not correlated with individual genetic diversity (*HL*: $F_{1,174} = 2.63$, $P = 0.107$; H_O: $F_{1,174} = 3.11$, $P = 0.080$).

DISCUSSION

We have found that physiological response to stress in fledgling Lesser Kestrels is higher in males and decreases with fledgling physical condition. Further, the heterophil/lymphocyte ratio is positively associated with offspring genetic diversity estimated at 11 polymorphic microsatellite loci. The negative association between heterophil/lymphocyte ratio and physical condition has been observed previously in other bird species (Suorsa *et al.* 2004, Alvarez *et al.* 2005, Lobato *et al.* 2005), suggesting that this parameter is sensitive to food shortage and may be a good indicator of nutritionally based physiological stress in nestlings (Gross & Siegel 1983, Maxwell & Robertson 1998, Moreno *et al.* 2002). It should be noted that pectoral thickness decreases sharply with food shortage, as indicated by the low values of pectoral thickness found in several chicks, which usually fall from their nests to the ground and often starve to death because they are generally not fed further by their parents (J.M. Aparicio unpubl. data). As is found in several other altricial bird species, our results suggest that some fledgling Lesser Kestrels are food limited and this is reflected in physiological stress during development. Analyses of specific leucocyte concentrations indicate that the association between fledgling pectoral thickness and heterophil/lymphocyte ratio may be driven by heterophil proliferation in individuals with poor physical condition (Davis *et al.* 2008).

Physiological response to stress was higher in males than in females and this effect was independent of physical condition. It should be noted that males and females within a brood do not differ in pectoral thickness before fledging (paired *t*-test, $t_{80} = 0.334$, $P = 0.739$), suggesting that the observed pattern is due to inherent sexual differences in the factors influencing heterophil/lymphocyte ratios. Further, Lesser Kestrels show no sexual size dimorphism (Aparicio & Cordero 2001) and thus differences between the sexes in heterophil/lymphocyte ratios do not result from the larger sex being less susceptible to poor rearing conditions (Lobato *et al.* 2008).

It is generally accepted that males are more susceptible to parasites due to physiological causes (interaction between hormones and immune system), and this could increase physiological response to stress particularly in that sex (Merino *et al.* 1998). However, we have not found a sex-biased pattern of infestations by the ectoparasite *Carnus haemapterus* (J. Ortego unpubl. data) and nestling Lesser Kestrels do not show patent infections by blood parasites before fledging (Ortego *et al.* 2008), suggesting that the observed pattern is not the result of sex-biased parasitism. This result may be due to differences between the sexes in their resistance to stress: for example, if males survive better than females under stressful conditions (Aparicio & Cordero 2001). In this context Merino *et al.* (2002) found that male Barn Swallows *Hirundo rustica* only showed higher levels of heat-shock proteins than females when comparing uninfected individuals, which also suggests that intersexual differences in physiological response to stress are mediated by intrinsic parameters other than parasites. Differences between the sexes in

levels of circulating corticosterone may be the underlying cause of the observed sexual pattern of heterophil to lymphocyte ratios, although to the best of our knowledge such differences in corticosteroids concentrations have not yet been reported in nestlings (Schwabl 1999, Sockman & Schwabl 2001).

The heterophil to lymphocyte ratio increased with offspring genetic diversity and this was not a consequence of heterozygosity at any particular locus, suggesting that genome-wide variability underlies the observed correlation as it occurs for other components of fitness (Ortego *et al.* 2007c, 2007d). Heterozygosity did not significantly correlate with the concentration of any leucocyte type, indicating that a redistribution of both the relative heterophil and lymphocyte concentrations, but not the proliferation of a particular leucocyte type, underlies the positive association between individual genetic diversity and heterophil/lymphocyte ratio. This association was surprising as it would be expected that fewer heterozygous individuals would show a higher physiological response to stress (i.e. a higher heterophil/lymphocyte ratio) if reduced genetic diversity decreases their competitive ability (Meagher *et al.* 2000, Tiira *et al.* 2003, 2006) or changes epistatic interactions between genes and/or increases the expression of recessive deleterious alleles and thus disrupts protein regulation (Mitton & Grant 1984, Kristensen *et al.* 2002, Pedersen *et al.* 2005). An explanation of our results may be linked with the effects of genetic diversity on levels of stress hormones, which in turn are known to directly affect heterophil/lymphocyte ratios (Davis *et al.* 2008). In this respect, previous experimental studies have clearly demonstrated that reduced genetic diversity decreases hormone levels, including testosterone and corticosterone (Kosowska & Zdrojewicz 1989, 1991). Given that corticosterone increases the number of heterophils and decreases lymphocyte counts (reviewed in Davis *et al.* 2008), a potential association between genetic diversity and circulating levels of corticosteroids may explain why genetically less diverse individuals show lower heterophil/lymphocyte ratios. Thus, we suggest that the observed association does not necessarily mean that more heterozygous individuals are more stressed but rather that a third unmeasured interaction between genetic diversity and hormone levels may have resulted in increased heterophil/lymphocyte ratios in more heterozygous offspring. In fact, increased genetic diversity is likely to reduce the susceptibility to environmentally induced stress, and future experimental studies analysing the link between stress tolerance and heterozygosity/inbreeding levels may help to clarify the observed association between physiological response to stress and individual genetic diversity (Keller & Waller 2002).

In summary, this study opens an interesting question about the effects of individual genetic diversity on physiological response to stress, which has been overlooked in previous research. Further studies directly analysing the association between levels of stress hormones and individual genetic diversity/inbreeding together with experiments based on laboratory species could help to resolve this interesting question in the future.

Primer sequences for microsatellite Fu1 and Fu2 were kindly provided by Jon H. Wetton (Forensic Science Service, UK). David Parkin, Rauri Bowie and two anonymous referees provided valuable comments on the manuscript. We manipulated and banded Lesser Kestrels under licence from the Spanish institutional authorities (Environmental Agency of the Community of Castilla-La Mancha and the Ringing Office of the Ministry of Environment) and we followed general ethical guidelines for animal welfare and nature conservation. This work received financial support from the projects: CGL2005-05611-C02-02/BOS (MEC), PAI05-053 (JCCM) and PCI08-0130-3954 (JCCM). The study was funded by a postdoctoral JAE-Doc (CSIC) contract (to J.O.) and a predoctoral fellowship from the Junta de Comunidades de Castilla-La Mancha/European Social Fund (to G.C.).

REFERENCES

Alvarez, F., Sanchez, C. & Angulo, S. 2005. Small, fat male Moorhens *Gallinula chloropus* are healthier. *Ardeola* **52**: 279–285.

Aparicio, J.M. 1997. Costs and benefits of surplus offspring in the Lesser Kestrel (*Falco naumanni*). *Behav. Ecol. Sociobiol.* **41**: 129–137.

Aparicio, J.M. & Bonal, R. 2002. Effects of food supplementation and habitat selection on timing of Lesser Kestrel breeding. *Ecology* **83**: 873–877.

Aparicio, J.M. & Cordero, P.J. 2001. The effects of the minimum threshold condition for breeding on offspring sex-ratio adjustment in the Lesser Kestrel. *Evolution* **55**: 1188–1197.

Aparicio, J.M., Ortego, J. & Cordero, P.J. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Mol. Ecol.* **15**: 4659–4665.

Arriero, E., Moreno, J., Merino, S. & Martinez, J. 2008. Habitat effects on physiological stress response in nestling Blue Tits are mediated through parasitism. *Physiol. Biochem. Zool.* **81**: 195–203.

Blas, J., Bortolotti, G.R., Tella, J.L., Baos, R. & Marchant, T.A. 2007. Stress response during development predicts fitness in a wild, long-lived vertebrate. *Proc. Natl Acad. Sci. U S A* **104**: 8880–8884.

Bolton, M., Monaghan, P. & Houston, D.C. 1991. An improved technique for estimating pectoral muscle protein condition from body measurements of live gulls. *Ibis* **133**: 264–270.

Calabuig, G., Ortego, J., Aparicio, J.M. & Cordero, P.J. 2008. Public information in selection of nesting colony by Lesser Kestrels: which cues are used and when are they obtained? *Anim. Behav.* **75**: 1611–1617.

Davis, A.K. & Maerz, J.C. 2008. Sex-related differences in hematological stress indices of breeding paedomorphic mole salamanders. *J. Herpetol.* **42**: 197–201.

Davis, G.S., Anderson, K.E. & Carroll, A.S. 2000. The effects of long-term caging and molt of single comb white Leghorn hens on heterophil to lymphocyte ratios, corticosterone and thyroid hormones. *Poult. Sci.* **79**: 514–518.

Davis, A.K., Cook, K.C. & Altizer, S. 2004. Leukocyte profiles in wild House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. *EcoHealth* **1**: 362–373.

Davis, A.K., Maney, D.L. & Maerz, J.C. 2008. The use of leucocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct. Ecol.* **22**: 760–772.

Foerster, K., Delhey, K., Johnsen, A., Lifjeld, J.T. & Kempenaers, B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* **425**: 714–717.

Gosler, A.G. & Harper, D. 2000. Assessing the heritability of body condition in birds: a challenge exemplified by the Great Tit *Parus major* L. (Aves). *Biol. J. Linn. Soc.* **71**: 103–117.

Gross, W.B. & Siegel, H.S. 1983. Evaluation of the heterophil lymphocyte ratio as a measure of stress in chickens. *Avian Dis.* **27**: 972–979.

Horak, P., Tegelmann, L., Ots, I. & Moller, A.P. 1999. Immune function and survival of Great Tit nestlings in relation to growth conditions. *Oecologia* **121**: 316–322.

Keller, L.F. & Waller, D.M. 2002. Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**: 230–241.

Kosowska, B. & Zdrojewicz, Z. 1989. Adrenal-medulla hormone response (adrenaline, noradrenaline, dopamine) to stress in rats of various inbreeding levels. *J. Anim. Breed. Genet.* **106**: 455–460.

Kosowska, B. & Zdrojewicz, Z. 1991. The role of genetic variability in the hormonal adaptative mechanism. 1. The effect of various homozygosity levels on the adrenal-cortex hormone concentrations (corticosterone and aldosterone) in the blood-serum of rats exposed to 2 types of stress. *J. Anim. Breed. Genet.* **108**: 147–153.

Krackow, S. & Tkadlec, E. 2001. Analysis of brood sex ratios: implications of offspring clustering. *Behav. Ecol. Sociobiol.* **50**: 293–301.

Kranendonk, G., Hopster, H., van Eerdenburg, F., van Reenen, K., Fillerup, M., de Groot, J., Korte, M. & Taverne, M. 2005. Evaluation of oral administration of cortisol as a model for prenatal stress in pregnant sows. *Am. J. Vet. Res.* **66**: 780–790.

Kristensen, T.N., Dahlgaard, J. & Loeschcke, V. 2002. Inbreeding affects Hsp70 expression in two species of *Drosophila* even at benign temperatures. *Evol. Ecol. Res.* **4**: 1209–1216.

Lobato, E., Moreno, J., Merino, S., Sanz, J.J. & Arriero, E. 2005. Haematological variables are good predictors of recruitment in nestling Pied Flycatchers (*Ficedula hypoleuca*). *Ecoscience* **12**: 27–34.

Lobato, E., Merino, S., Morales, J., Tomas, G., Martínez-De La Puente, J., Sánchez, E., Garcia-Fraile, S. & Moreno, J. 2008. Sex differences in circulating antibodies in nestling pied flycatchers *Ficedula hypoleuca*. *Ibis* **150**: 799–806.

Martínez-Padilla, J., Martinez, J., Davila, J.A., Merino, S., Moreno, J. & Millan, J. 2004. Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian Kestrel nestlings. *Funct. Ecol.* **18**: 426–434.

Maxwell, M.H. & Robertson, G.W. 1998. The avian heterophil leucocyte: a review. *Worlds Poult. Sci. J.* **54**: 155–178.

Meagher, S., Penn, D.J. & Potts, W.K. 2000. Male-male competition magnifies inbreeding depression in wild house mice. *Proc. Natl Acad. Sci. U S A* **97**: 3324–3329.

Merino, S., Martinez, J., Barbosa, A., Moller, A.P., de Lope, F., Perez, J. & Rodriguez-Caabeiro, F. 1998. Increase in a heat-shock protein from blood cells in response of nestling House Martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia* **116**: 343–347.

Merino, S., Martinez, J., Moller, A.P., Barbosa, A., de Lope, F. & Rodriguez-Caabeiro, F. 2002. Blood stress protein levels in relation to sex and parasitism of Barn Swallows (*Hirundo rustica*). *Ecoscience* **9**: 300–305.

Merino, S., Moreno, J., Tomas, G., Martinez, J., Morales, J., Martinez-De La Puente, J. & Osorno, J.L. 2006. Effects of parental effort on blood stress protein HSP60 and immunoglobulins in female Blue Tits: a brood size manipulation experiment. *J. Anim. Ecol.* **75**: 1147–1153.

Mitton, J.B. & Grant, M.C. 1984. Associations among protein heterozygosity, growth-rate, and developmental homeostasis. *Ann. Rev. Ecol. System.* **15**: 479–499.

Moreno, J., Merino, S., Martinez, J., Sanz, J.J. & Arriero, E. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience* **9**: 434–439.

Morici, L.A., Elsey, R.M. & Lance, V.A. 1997. Effects of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. *J. Exp. Zool.* **279**: 156–162.

Nesje, M., Roed, K.H., Lifjeld, J.T., Lindberg, P. & Steen, O.F. 2000. Genetic relationships in the Peregrine Falcon (*Falco peregrinus*) analysed by microsatellite DNA markers. *Mol. Ecol.* **9**: 53–60.

Newton, S.F. 1993. Body condition of a small passerine bird – ultrasonic assessment and significance in overwinter survival. *J. Zool., Lond.* **229**: 561–580.

Ortego, J. & Espada, F. 2007. Ecological factors influencing disease risk in Eagle Owls *Bubo bubo*. *Ibis* **149**: 386–395.

Ortego, J., Aparicio, J.M., Calabuig, G. & Cordero, P.J. 2007a. Increase of heterozygosity in a growing population of Lesser Kestrels. *Biol. Lett.* **3**: 585–588.

Ortego, J., Gonzalez, E.G., Sanchez-Barbudo, I., Aparicio, J.M. & Cordero, P.J. 2007b. New highly polymorphic loci and cross-amplified microsatellites for the Lesser Kestrel *Falco naumanni*. *Ardeola* **54**: 101–108.

Ortego, J., Calabuig, G., Cordero, P.J. & Aparicio, J.M. 2007c. Egg production and individual genetic diversity in Lesser Kestrels. *Mol. Ecol.* **16**: 2383–2392.

Ortego, J., Aparicio, J.M., Calabuig, G. & Cordero, P.J. 2007d. Risk of ectoparasitism and genetic diversity in a wild Lesser Kestrel population. *Mol. Ecol.* **16**: 3712–3720.

Ortego, J., Cordero, P.J., Aparicio, J.M. & Calabuig, G. 2008. Consequences of chronic infections with three different avian malaria lineages on reproductive performance of Lesser Kestrels (*Falco naumanni*). *J. Ornithol.* **149**: 337–343.

Pedersen, K.S., Kristensen, T.N. & Loeschcke, V. 2005. Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster* – Hsp70 expression and fitness. *J. Evol. Biol.* **18**: 756–762.

SAS Institute. 2004. *SAS/STAT 9.1 User's Guide*. Cary, NC: SAS Institute Inc.

Schwabl, H. 1999. Developmental changes and among-sibling variation of corticosterone levels in an altricial avian species. *Gen. Comp. Endocrinol.* **116**: 403–408.

Sears, J. 1988. Assessment of body condition in live birds: measurements of protein and fat reserves in the Mute Swan, *Cygnus olor*. *J. Zool., Lond.* **216**: 295–308.

Singer, J.D. 1998. Using SAS PROC MIXED to fit multilevel models, hierarchical models, and individual growth models. *J. Educat. Behav. Stat.* **23**: 323–355.

Sockman, K.W. & Schwabl, H. 2001. Plasma corticosterone in nestling American kestrels: effects of age, handling stress, yolk androgens, and body condition. *Gen. Comp. Endocrinol.* **122**: 205–212.

Suorsa, P., Huhta, E., Nikula, A., Nikinmaa, M., Jantti, A., Helle, H. & Hakkarainen, H. 2003. Forest management is associated with physiological stress in an old-growth forest passerine. *Proc. R. Soc. Lond. B* **270**: 963–969.

Suorsa, P., Helle, H., Koivunen, V., Huhta, E., Nikula, A. & Hakkarainen, H. 2004. Effects of forest patch size on physiological stress and immunocompetence in an area-sensitive passerine, the Eurasian Treecreeper (*Certhia familiaris*): an experiment. *Proc. R. Soc. Lond. B* **271**: 435–440.

Tiira, K., Laurila, A., Peuhkuri, N., Piironen, J., Ranta, E. & Primmer, C.R. 2003. Aggressiveness is associated with genetic diversity in landlocked salmon (*Salmo salar*). *Mol. Ecol.* **12**: 2399–2407.

Tiira, K., Laurila, A., Enberg, K., Piironen, J., Aikio, S., Ranta, E. & Primmer, C.R. 2006. Do dominants have higher heterozygosity? Social status and genetic variation in brown trout, *Salmo trutta*. *Behav. Ecol. Sociobiol.* **59**: 657–665.

Vleck, C.M., Vertalino, N., Vleck, D. & Bucher, T.L. 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adelie Penguins. *Condor* **102**: 392–400.

Received 19 December 2008;
revision accepted 24 April 2009.