MHC Diversity and Differential Exposure to Pathogens in Two Congeneric Birds

MIGUEL ALCAIDE, JESÚS A. LEMUS*, GUILLERMO BLANCO*, JOSÉ L. TELLA, DAVID SERRANO, JUAN J. NEGRO, AIRAM RODRÍGUEZ and MARINO GARCÍA-MONTIJANO**

*Estación Biológica de Doñana (CSIC), Pabellón de Perú, Avda. Maria Luisa s/n, 41013, Sevilla, Spain, *Departamento de Ecología Evolutiva, Museo de Ciencias Naturales (CSIC) J. Gutiérrez Abascal 2, 28006, Madrid, **GIR diagnostics SL. C/ Ontanilla, 2, Torrelaguna, 28180, Madrid.*

Running Title: Pathogens and MHC diversity in kestrels

Author contributions

Conceived and designed the work: MA, JJN, JL, GB, DS, JLT. Conducted field work: AR, JAL, GB, DS, JLT, MGM. Performed genetic analyses: MA. Performed pathogen determinations: JAL, MGM. Analyzed the data: MA, GB, JLT. Contributed reagents/materials/analysis tools: JJN, GB, MGM. Wrote the paper: MA, JLT, GB.

Background. Extensive genetic variation at the Major Histocompatibility Complex (MHC) is believed to have evolved as a means of fighting off a broad spectrum of infectious diseases. Here, we surveyed a whole community of pathogens (N=35 taxa) in two phylogenetically related and sympatric raptorial birds (Eurasian and lesser kestrels) differing in ecology, life history traits and expected levels of pathogen exposure.

Principal findings. Although specific host traits may explain the differential infection by particular pathogens, overall pathogen diversity, richness and prevalence were higher in Eurasian than in lesser kestrels. Accordingly, we found a higher number of alleles (64 vs. 49) as well as more divergent MHC class I and class II haplotypes in Eurasian than in lesser kestrels. Detailed analyses of amino acid diversity showed that differences were only statistically significant for those functionally important codons comprising the antigen binding sites. The lack of significant differences at 8 microsatellite markers allowed discarding the confounding effect of effective population size on genetic diversity. The lowest pathogen burdens and the smallest but still quite divergent set of MHC sequences were found in Eurasian kestrels from the Canary Islands (16 alleles), where the rates of allele fixation at MHC loci seem to have occurred faster than at microsatellites.

Conclusions/Significance. A variety of ecological requirements and life history traits of hosts are shaping in a complex way their wide community of pathogens. This study shows correlated estimates of pathogen burdens and MHC diversity in birds which illuminates the role of pathogen diversity and abundance in

shaping MHC variation. Crucial for the triggering of adequate adaptive immune responses, MHC variability may constitute a good predictor of host susceptibility to infections. Thus, limited MHC variation derived from a historical low exposition to pathogens may become counterproductive in a present context of global change and emerging infectious diseases.

INTRODUCTION

Genetic diversity at functionally important genes such as those belonging to the major histocompatibility complex (MHC) is widely believed to influence the evolutionary and adaptive potential of populations and species [1,2]. This multigene family plays a central role in the immune system of vertebrates [3]. In particular, MHC genes code for cell-surface glycoproteins that bind foreign peptides for their presentation to specialized cells of the immune system, which subsequently trigger adequate immune responses. MHC class I molecules bind peptides derived from the processing of intracellular pathogens, such as viruses and some protozoa, and promote the destruction of the antigen-presenting cell. On the other hand, the recognition of foreign antigens bound to MHC class II molecules stimulates antibody production against bacterial or parasite proteins. Genetic variation at MHC genes largely determines the number of foreign antigens an individual is capable of responding to, and thus, MHC diversity is thought to decisively influence individual fitness and long-term persistence of populations [4]. The selective pressures imposed by pathogens have turn MHC genes into the most polymorphic coding loci described so far [5], and consequently, this huge variability has attracted evolutionary biologists' attention. The intensity of selection is especially significant in those amino acid positions belonging to the peptide-binding region (PBR), a highly variable extracellular groove that determines the specificity of MHC molecules. Balancing selection and MHC-dependent mate choice are among the most widely accepted evolutionary mechanisms aimed at maintaining the high levels of MHC polymorphism needed to counteract pathogen and parasite-mediated selective pressures [1,2]

Whether extraordinary high levels of MHC polymorphism are intended to cope with a broad array of potential infections, the strength of diversifying selection at MHC loci is expected to be driven by the richness and virulence of parasite and pathogen taxa to which hosts are exposed, which in turn, should be related to both host's life histories and ecological conditions. In this respect, it has been documented in the literature the influence of temperature clines in the world-wide distribution and virulence of parasites [6]. Patterns of habitat use and range distribution are thought to determine the extent and cohabitation period of some host-parasite interactions [7]. In addition, species that only thrive within a range of environmental conditions are believed to hold lower but more specialized parasite and pathogen burdens than generalist species with a broad tolerance to environmental conditions [8]. Migratory species are commonly exposed to at least two different parasite and pathogen faunas during their annual cycle [9], whilst resident species only have to face one. High prevalence of parasites in socially-breeding species has been attributed to high transmission rates in the colonies [10]. An axis of body size, developmental period and life span of hosts has been positively related to their immunocompetence [11] and, therefore, to lower infection rates. Risks of infections are also expected to differ among species with different feeding habits given that prey items constitute a potential source of pathogens and parasites [12,13].

Despite a growing interest in the understanding of the sources of variability in pathogen pressure driving MHC evolution, studies associating environmental and ecological factors with MHC diversity in natural populations

145 are notably few [14-17]. In birds, even though it is widely recognised that
146 pathogen pressure greatly varies among species with different ecologies and
147 life histories, the majority of research efforts have focused on inter-specific
148 comparative studies of immunocompetence surrogates, such as the size of
149 immune organs [18] and particular immune responses [11], without relating
150 them to actual parasite and pathogen burdens and MHC evolution. In addition,
151 the study of MHC variation in avian species has been mainly put in context of
152 different demographic histories [19,20], local adaptations [21,22] or MHC-
153 disassortative mating patterns [23-25]. The few examples linking avian MHC
154 diversity to resistance/susceptibility to infectious diseases have dealt so far with
155 single host-parasite associations [26,27].
156

157 We present here one of the very few studies that simultaneously
158 investigates variability at both MHC class I and class II genes in relation to a
159 whole community of pathogens and parasites, including viruses, bacteria, fungi,
160 protozoan and helminths (thereafter termed pathogens for simplicity), in wild
161 populations of two bird species with contrasting life histories and ecological
162 requirements. We investigated three subspecies of the Eurasian Kestrel *Falco*
163 *tinnunculus* and the phylogenetically related Lesser Kestrel *Falco naumanni*
164 [28]. While the Lesser Kestrel is a habitat-specialist, estenophagous, colonial
165 and migratory falcon, the sympatric European subspecies of the Eurasian
166 Kestrel *Falco t. tinnunculus* is primarily considered a cosmopolitan territorial
167 breeder, euriphagous, sedentary and habitat generalist species [29]. We
168 expected that differences in ecology and life histories between these two
169 species (see details in Table S1) would determine a differential exposure to

pathogens, according to the hypotheses outlined above, and that these differences would translate to MHC variability. Since our compiled hypotheses predict alternative pathogen pressure outcomes (in terms of diversity, richness and/or prevalence, see Table 1), and different kinds of pathogens may greatly vary in their virulence and selective forces on hosts, it is difficult to anticipate a single clear prediction on which species would show greater MHC polymorphism (Table 1). In addition, we also sampled two island subspecies of the Eurasian Kestrel, *Falco t. dacotiae and Falco t. canariensis*, which were expected to hold lower MHC diversity and prevalence of infections (Table 1) because of the demographic and genetic constraints typically associated with insularity, which affects both communities of infectious agents and their hosts [8,30]. Finally, MHC variability was examined in conjunction with patterns of neutral genetic variation (microsatellites) to discard the confounding effects of effective population size.

RESULTS

Patterns of pathogens infection

Results from the pathogen screening are detailed in Table S2, while diversity, richness, prevalence and individual richness estimates are compiled in Table 2. The highest values of these pathogen burden indicators were found in adult Eurasian kestrels sampled in the continent (*F. t. tinnunculus*), greatly exceeding those found in the sympatric lesser kestrel (*F. naumanni*). Fledglings showed lower values than adults, with slight differences between the two species. Adult Eurasian kestrels sampled from the island subspecies (*F.t. dacotiae* and *F.t.*

195 *canariensis*) showed the lowest pathogen burdens, comparable to continental
196 nestlings, and markedly below that of continental Eurasian kestrel adults.

197

198 A Categorical Principal Component Analysis (CatPCA) allowed us to
199 assess the associations of pathogens differentially infecting species/subspecies
200 and age classes. The CatPCA yielded four dimensions with an eigenvalue
201 greater than 1 that accounted for 64% of the variance (Table 3). The first
202 dimension (D1) revealed high viral and bacterial loads and hence defines a
203 gradient of infection combining the prevalence and number of species of these
204 pathogens. The second dimension (D2) showed high values for haematozoa
205 and *Trichomonas gallinae* and, therefore, it defines a gradient of protozoan
206 infection. The third (D3) and fourth (D4) dimensions included high values for
207 *Candida albicans* (the only fungi identified) and *Mycoplasma* sp., respectively
208 (Fig. 1). Factor scores of the individual birds in each dimension showed several
209 statistical differences between groups (species/subspecies) and age-classes
210 (Table 4). Continental Eurasian kestrels were more infected by viruses and
211 bacteria (D1) than lesser kestrels, both in fledglings and adults (Fig. 1).
212 Contrarily, the infection by protozoans (D2) was higher in lesser than in
213 Eurasian kestrels (Fig. 2), although the significance of the latter was much lower
214 than in the case of viruses and bacteria. Infection mostly headed by *C. albicans*
215 (D3) and *Mycoplasma* sp. (D4) showed differences between species only for
216 nestlings, with higher incidence in Eurasian and lesser kestrel respectively (Fig.
217 2). Regarding island effects, adult Eurasian kestrels from the continent showed
218 higher infections by viruses and bacteria (D1) and *Mycoplasma* sp. (D 4) than
219 those from the Canary Islands (Fig. 1).

When looking at infections by particular pathogens, univariate comparisons of prevalence estimates showed many significant differences between adults and nestlings of both Eurasian and lesser kestrels in the continent, adults always showing higher prevalences than fledglings (Table S3). Fledgling Eurasian kestrel showed significant higher prevalences of *Leucocytozoon*, *C. albicans*, *Campylobacter* sp., adenovirus and reovirus than lesser kestrels, while the opposite was found for *T. gallinae*, enterotoxigenic *E. coli*, *Mycoplasma* sp. and influenzavirus IH7 (Table S3). Adult Eurasian kestrels showed a higher prevalence of *P. multocida*, enterotoxigenic *E. coli* and paramixovirus than lesser kestrels, while the latter was more frequently infected by West-Nile virus. Multiple pathogens showed higher prevalences in continental than in kestrels from the Canary Islands (Table S3). Only *Plasmodium* sp. and West-Nile virus infected more frequently island kestrels.

Genetic diversity at microsatellites and MHC loci

Detailed polymorphism statistics at microsatellites and MHC loci are summarized in Table 5. Average microsatellite diversity per individual was not significantly different after comparing the two species of kestrels living in the continent. Average homozygosity by loci was 0.1725 for lesser kestrels and 0.1625 for Eurasian kestrels (t=-0.3797, df=48, P=0.71). Conversely, island kestrels showed significant lower genetic diversity than mainland Eurasian kestrels (Homozygosity by loci estimates: 0.26 vs 0.1625, respectively; t=3.44, df=28, P=0.001). Island subspecies were clumped together given that we did not find significant differences at both neutral (F_{ST}<0) and adaptive loci (K_{ST}<0).

Kestrel MHC sequences are deposited in GenBank (Acc No. EU120698-EU120722, EF370767-370788 and EU107667-EU107746, see also FigS1 and FigS2).

MHC amino acid diversity per site ranged from 0.05 (conserved site) to 0.22 (the most polymorphic site) (see Fig. 2). After comparing paired values of the amino acid diversity parameter *d* at each PBR codon position for both class I and class II loci, we found statistically significant evidence for higher amino acid diversity within the mainland population of Eurasian kestrels than in lesser kestrels (Wilcoxon matched-pairs test: W+=478.5, W-=187.5, N=36, P=0.023). On the contrary, amino acid diversity differences at non-PBR codons was not significant (Wilcoxon matched-pairs test: W+=199.5, W-=265.5, N=30, P=0.50). A similar analysis comparing continental and insular populations is not adequate because of the lack of evolution in sympatry and the influence of founder events during island colonization [28].

DISCUSSION

This is one of the very few studies that associates detailed sequence polymorphism at both class I and class II MHC genes with extensive surveys of pathogen communities in wild animal populations. We found that the number of MHC alleles as well as the extent of genetic divergence between MHC haplotypes was positively related to pathogen burdens in two congeneric and sympatric birds, the Eurasian and the lesser kestrel. Even though the continental population of the former in our study area practically doubles that of

the latter [31], we controlled for the confounding effect of effective population size through the analysis of 8 polymorphic and presumably neutral microsatellite markers. In addition, comparisons of amino acid diversity were only significant when restricting the analysis to those functionally important codons belonging to the PBR (Fig. 2). Importantly, whilst the majority of studies have extrapolated the positively selected amino acid sites of the human MHC [17,32], we used detailed analyses identifying positively selected amino acid sites within single MHC genes in kestrels [22,33]. Our results therefore suggest a higher incidence of diversifying selection acting on MHC genes of the Eurasian kestrel in agreement with its higher exposure to pathogens.

Even though one of most cited implication underlying MHC theory outlines the role of infectious agents in driving diversifying selection at functionally important loci [1,2,34], few studies have demonstrated clear positive correlations between MHC diversity and pathogen species richness in wild populations so far. For instance, Prugnolle and co-workers [15] showed that genetic diversity at the HLA-B gene was notably influenced by local diversity of intracellular pathogens in human populations. Göuy de Bellocq and colleagues [35] has recently accomplished an interspecific survey documenting a positive correlation between helminth diversity and MHC class II polymorphism in rodents. In a similar way, a recent study in the Atlantic salmon [17] reports a positive correlation between the temperature of rivers, which affects the richness and virulence of pathogen communities, and MHC class II diversity across a latitudinal gradient in Eastern Canada. Apart from a few studies cited above, the present work on kestrels is one of the first that have also taken into

296 account the role of neutral evolutionary forces linked to demographic processes
297 and population structure [2].

298

299 Several ecological factors and life history traits may explain different risks
300 of infection among host species. The majority of studies to date have however
301 centred on a single or reduced group of pathogenic species to test different
302 hypotheses related to such variability [36-38]. Our approach at the pathogen
303 community level suggests that several hypotheses acting together in a complex
304 way, rather than disentangling hypotheses, may explain overall differences in
305 pathogen burdens between congeneric kestrels. While fledglings of both
306 Eurasian and lesser kestrels showed similar and low pathogen burdens,
307 probably because of reduced opportunities for pathogen transmission from
308 hatching to fledging time, the diversity, richness, and prevalence of pathogens
309 were markedly higher in adult Eurasian than in sympatric adult lesser kestrels.
310 The larger exposure of Eurasian kestrels to viruses and bacteria (D1 in Fig. 1),
311 which encompass the most virulent pathogens among the wide array of taxa we
312 surveyed, might have decisively contributed to its larger MHC diversity.
313 Certainly, the truly cosmopolitan character of the Eurasian kestrel may have
314 increased the diversity of infectious agents this species has been exposed to
315 during its evolutionary history [7], whilst the lesser kestrel became a steppe-
316 specialist falcon with more restricted geographic range and habitat uses
317 presumably limiting pathogen interactions. In fact, the bacteria *Pseudomonas*
318 *aeruginosa* and *Mycobacterium avium*, herpesviruses, as well as intestinal
319 parasites of the genus *Ascaridia*, *Isospora*, and *Cyrnea* were exclusively found
320 infecting Eurasian kestrels (Table S2). By contrast, there was no infection found

in lesser kestrels that was not detected in Eurasian kestrels either. Moreover, the broader feeding spectrum displayed by Eurasian kestrels may decisively have increased risk of infections when compared to the insect-specialist lesser kestrel. This phenomenon may explain the higher prevalences in the former of *Campylobacter* sp*., P. multocida* and paramixovirus, all of them infections which may be transmitted from mammals to birds of prey [12,9,39]. On the other hand, lesser kestrels were more infected with *Mycoplasma* sp. and *Trichomonas gallinae*, whose horizontal transmission among nestlings is enhanced by the close proximity of nests and even by the frequent nest-switching of fledglings in the colonies [40]. The usual presence of other species commonly hosting these pathogens, such as the domestic pigeon *Columba livia* [41,12,42], may explain higher prevalences of these infections in lesser kestrel colonies as well. Finally, the higher prevalence of West Nile virus and IH7 in lesser kestrels may be reasonably related to their migratory behaviour [9,43-47].

In addition to pathogen pressure, the degree of genetic exchange is also expected to influence the extent of local adaptations in open populations. A recent study conducted for lesser kestrels has reported significant isolation by distance patterns across the Western Palearctic when analysing both adaptive (MHC class II polymorphism) and neutral (microsatellites) data [22]. Thus, restricted gene flow may favour directional selection of some alleles or allelic lineages over others in this species. Conversely, the population of Eurasian kestrels in the Western Palearctic has shown high levels of genetic uniformity after analysing the same microsatellite set [48]. This finding suggests comparably higher levels of gene flow between Eurasian kestrel populations, a

346 fact that would limit the loss of MHC alleles because of local selection and
347 genetic drift. Although restricted gene flow would be in agreement with a
348 comparably lower number of MHC alleles in the lesser kestrel, the stronger
349 genetic hallmark of diversifying selection at the PBR of the Eurasian kestrel
350 could not be explained by neutral evolutionary forces but because of pathogen-
351 mediated selective pressures.

352

353 Finally, the comparison between mainland and insular subspecies of the
354 Eurasian kestrel constitutes the strongest support for the role of the diversity
355 and amount of infectious agents in maintaining high levels of MHC variability.
356 Our surveys of pathogens in islands failed to detect up to 14 different bacterial,
357 viral, and parasitic infections commonly hosted by continental kestrels.
358 Moreover, pathogen prevalences were significantly higher in continental birds in
359 six out of eight comparisons involving infections shared by island kestrels. On
360 the other hand, while neutral selective forces such as population bottlenecks
361 and founder events have provoked the loss of about 25% of microsatellite
362 diversity in the Canary Islands, the fixation rates at MHC sequences seem to
363 have occurred three times faster (Table 5). These results would highlight the
364 inadequacy of using neutral markers as surrogates for genetic variation in
365 fitness-related loci in some situations [49,50]. Since diversifying selection might
366 be constrained by locally impoverished pathogen communities, we believe that
367 natural selection has promoted the fixation of the most efficient MHC alleles.
368 However, and in agreement with a study conducted for great reed warblers [19],
369 selection has preserved high genetic divergence. The average number of
370 nucleotide differences between unique alleles in island subspecies has

371 increased in the case of class II alleles but not in the case of class I alleles
372 (Table 5). These genetic data are congruent with a comparably higher incidence
373 of bacteria species in the Canary Islands (4 out of 7 species isolated in
374 mainland kestrels) in relation to viral infections (only 2 out of 7 types of viral
375 infections isolated in mainland kestrels).

376

377 In conclusion, the results derived from this study support correlated
378 levels of MHC diversity and pathogen burdens in wild populations of kestrels.
379 Geographic range, patterns of habitat occupancy, migratory behaviour and
380 trophic diversity are among the most important factors delimiting patterns of
381 pathogen exposure in the species and subspecies we investigated. In this
382 regard, local adaptations to the typically impoverished pathogen communities of
383 insular ecosystems [8,30] may explain the low MHC polymorphism that we
384 found in island subspecies. Nevertheless, our comparative study has only dealt
385 with three related taxa, and more research should therefore be encouraged to
386 confirm whether reduced MHC diversity may emerge as an additional cost of
387 specialization. Importantly, pathogens are spreading worldwide due to human
388 transport, invasive hosts, land-use and climate change, increased
389 connectedness and globalisation [51], to the point that emergent diseases are
390 among the major current threats to global biodiversity [52,53]. One of the main
391 barriers to invasive pathogens is the host's immune system [54] and, in
392 agreement with the already documented massive extinctions in islands [55], the
393 most dramatic consequences may be therefore derived from the introduction
394 and spread of alien pathogens throughout immunologically naïve species
395 [56,57]. Consequently, there is the need to find the ways to predict the

occurrence and impact of emerging infectious diseases [51]. Accordingly with the results presented in this study, we expect the survey of MHC variability will emerge as a valuable compiling tool in this respect.

MATERIALS AND METHODS

Study Species and Populations

We sampled continental Eurasian and lesser kestrels from different locations in Spain and the two island subspecies of Eurasian Kestrels inhabiting the Canarian Archipelago (Fig. 3). The Spanish continental population of Lesser Kestrels is estimated at 12,000-20,000 breeding pairs [58], whilst that of the Eurasian Kestrel is believed to be constituted by 25,000-30,000 breeding pairs [31]. Insular subspecies are represented by 4,000-5,000 breeding pairs in *Falco t. canariensis* and about 400 breeding pairs in *Falco t. dacotiae* [59].

Biological samples for genetic analyses and pathogen surveys

For MHC and microsatellite determination, we genotyped 25 Lesser Kestrels hatched in large colonies (>10 breeding pairs), 25 Eurasian Kestrels raised by solitary breeding pairs, and 25 island kestrels including both adults and nestlings. All individuals come from different nests and were therefore presumably unrelated given the low rates of extra-pair paternity and intraspecific brood parasitism even in the colonial lesser kestrel [60]. About 8-9 continental kestrels were sampled at each of the geographic locations indicated by asterisks in Figure 3. Twelve island kestrels were sampled in Fuerteventura and 13 birds were sampled in Tenerife (Fig. 3). About 100 µl of blood preserved in 96% ethanol were digested by incubation with proteinase K for at least 3

hours. DNA purification was carried out by using 5M LiCl organic extraction method with chloroform-isoamylic alcohol (24:1) and further DNA precipitation using absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol, and later stored at –20º C in 0.1-0.2 ml of TE buffer.

For pathogen surveys, adult kestrels were captured on nests or using bal-chatri traps, while nestlings were sampled on nests when they were close to fledge (i.e., ca. 25-days old). Sampled locations are shown in Figure 3. As for genetic analyses, we sampled only one fledgling per nest in colonies of lesser kestrels and unrelated breeding pairs in the case of Eurasian kestrels. Only adult birds from the Canary Islands were obtained (see Table S2 for the number of individuals sampled for each species and subspecies). We collected oral and cloacae swabs as well as faecal samples. About 0.1 ml of blood taken from the brachial vein was stored in absolute ethanol for screening pathogens through PCR. Two blood smears were immediately taken and later fixed in the laboratory using absolute ethanol. Individuals for the genetic and species richness surveys were sampled in the same populations during a short period of time (2002-2006). Hence, we did not expect artefacts derived from the analysis of birds which had not been included in the genetic survey. Furthermore, recent analyses of population structure at MHC class II loci in Lesser Kestrels have shown high levels of genetic uniformity throughout the Iberian Peninsula [22].

Microsatellite and MHC genotyping

Eight microsatellite markers (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107 and Cl347) [61], [48] were amplified using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl

448 reaction contained 0.2 units of Taq polymerase (Bioline), 1x PCR manufacturer
449 supplied buffer, 1.5 mM MgCl2 , 0.02% gelatine, 0.12 mM of each dNTP, 5
450 pmol of each primer and, approximately, 10 ng of genomic DNA. F-Primers
451 were 5'-end labelled with HEX, NED or 6-FAM. Amplified fragments were
452 resolved on an ABI Prism 3100 Genetic Analyser and further scored using the
453 programmes Genotyper and GeneMapper (Applied Biosystems).
454
455 We amplified complete exon 2 sequences of an MHC class II B gene and
456 complete exon 3 sequences of a classical MHC class I gene [33, 62] using the
457 following PCR profile: 1 cycle of 4 min at 94ºC, 35 cycles of 40s at 94ºC, 40s at
458 54ºC (for class I loci) or 56ºC (for class II loci), 40s at 72º C and finally, 4 min at
459 72ºC. Each 25 µl reaction contained 0.4 units of Taq polymerase (Bioline), 1x
460 PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP,
461 10 pmol of each primer, 5% DMSO and, approximately, 25 ng of genomic DNA.
462 Investigation of variation at MHC loci requires separating the different PCR
463 amplification products because of individuals are likely to be heterozygous.
464 After PCR clean-up in Microcon centrifuge tubes (Millipore), PCR products were
465 cloned into bacterial plasmid using the PGEM-T easy vector system II
466 (Promega). Clones were screened for the expected insert size in 1.5 % agarose
467 gels by running a second PCR with M13 primers. Six to eight positive clones
468 per individual were selected at random for sequencing analysis. Sequencing
469 reactions were carried out using the Big Dye 1.1 Terminator technology and
470 labelled fragments were subsequently resolved in a 3100 automated sequencer
471 (Applied Biosystems).
472
473 **Estimates of genetic diversity at neutral and adaptive loci**

Individual microsatellite diversity was measured as a means of homozygosity by loci estimates [63] and compared using un-paired t-tests. MHC sequences were aligned and edited using BioEdit 7.0.5.2 [64]. Those cloned sequences differing in no more than 2 base pairs with respect to a redundant sequence were considered PCR artefacts or base misincorporations during bacterial replication. Since recombination of cloned PCR products is an additional source of artefacts [65], direct sequencing of uncloned PCR products was used to check for agreement of polymorphic sites with cloned sequences. All alleles found only in one individual were verified by performing a second typing of that individual. Polymorphism statistics were generated using the software DNAsp ver 4.20 [66].

MHC amino acid diversity for both species of continental kestrels was estimated for PBR and non-PBR codons separately by means of the diversity index *d* calculated using the programme DIVAA [67]. Conserved regions are characterised by low values of *d*, whilst highly polymorphic positions display high values of *d*. A discrepancy between PBR and non-PBR diversity would provide evidence concerning the intensity of selection acting specifically on antigen binding sites of MHC molecules. Putative amino acid sites conforming the PBR of MHC class I and class II molecules in kestrels, i.e. those displaying strong positive selection via an excess of non-synonymous over synonymous nucleotide substitutions, were previously identified using maximum likelihood and Bayesian methods [22,33,62].

Pathogen determination

For each individual, we determined the presence/absence of 35 organisms recognised as potential avian pathogens, including protozoa, bacterial, viral, fungal, haematozoan and helminths species (Table S2). We therefore covered almost the whole pathogenic community, with the only exception of ectoparasites. The two most common ectoparasites of kestrels are the haematophagous louse flies (Hippoboscidae: *Ornithophila gestroi*) and Carnus flies (*Carnus hemapterus*), which we were unable to correctly sample because they infect nestlings at a very narrow period of growth [68]. Although unusual, large infestations of louse flies may affect host fitness [69], the low prevalence and abundance of both louse and *Carnus* flies in kestrels suggests weak effects on their hosts [68,70]. On the other hand, the feather-eating Mallophaga (Insecta) is very rare in kestrels (authors, unpublish. data), and feather mites (Acari) are considered mutualistic rather than pathogenic [71]. Therefore, the absence of these scarce ectoparasites from our pathogen surveys should not decisively affect predictions on MHC diversity.

Pathogenic oral fungi *(Candida albicans)* were grown by incubating at 37ºC for 48 hours on standard fungical media composed of Agar Sabouraud. This fungus was also determined by PCR from blood samples [72] when samples from the oral cavity were not available. Pathogenic oral *(Pasterella multocida)* and cloacal bacteria *(Salmonella sp., Campylobacter jejuni,* enterotoxigenic *Escherichia coli* and *Pseudomonas aeruginosa)* were cultured on 5% sheep blood agar, chocolate agar and McConkey agar to avoid *Proteus sp*. overgrowth. Plates were incubated at 37ºC using both normal atmospheric and microaerophilic (10% CO_2) conditions during 24 hours. Suspected colonies

were subsequently subcultured on appropriate medium and identified using multi-substrate identification strips (API 20 E; BioMerieux) (see [73] for details). When microbiological samples were not available, the presence of pathogenic bacteria was determined via PCR from blood samples using commercial primers kits (BAX Real time PCR Assay, Dupont) as well as standard methods [74-76]. *Campylobacter* colonies were identified through PCR-RFLP of the flagellin gene A [77]. Samples from the same individuals were concurrently tested with microbiology standard culture procedures and PCR approaches, with no contradictions among samples. The presence of *Chlamydia psittaci* in blood was determined using PCR-based methods described in [78], while *Mycoplasma sp*. was determined as described in [79] and [42]. The presence of poxvirus, the paramyxovirus causing the Newcastle disease, the serotypes H5, H7 and H9 of the avian influenza, adenovirus, circovirus, herpesvirus, polyomavirus, reovirus and West Nile virus was determined following the PCR-based methods available in the literature [80-88].

Blood parasites (Haematozoa) were checked through traditional microscopic screening of blood smears [7] and PCR-based methods [89], [90] to increase the accuracy of detection [91], [92]. We looked for the protozoa *Trichomonas gallinae* in the crop mucosa collected with swabs and stored in warm sterile physiologic solution as well as through PCR detection [93]. Fresh faecal samples were examined for coccidian species (Protozooa) by oocyst sporulation with 2.5% potassium dichromate during fourteen days [94], followed by zinc sulfate flotation. For the detection of helminths eggs in faeces (trematodes, acantocephalans, cestodes and nematodes) we used the flotation

549 method with zinc sulphate solution as well as the slide direct examination
550 procedure [95,96].
551
552 Host sample sizes for age classes and species/subspecies were usually
553 large enough (> 15 individuals, range 17-244 except in two cases, see Table
554 S2) to allow obtaining reliable estimates of pathogen prevalences, and thus they
555 were suitable for statistical comparisons [97].
556
557 **Analyses of variability in pathogen infection**
558 As a first exploratory overview, we calculated the richness (number of pathogen
559 species), diversity (using the Shannon-Wiener index), prevalence (percentage
560 of birds infected by at least one pathogen) and individual pathogen richness
561 (number of pathogen species per individual) for each species, subspecies and
562 age-class of kestrels sampled. Going deeper, we then tested for differences in
563 the prevalence of each pathogen between continental lesser and common
564 kestrels (considering nestlings and adults separately), as well as between
565 continental and insular adult common kestrels (pooling both island subspecies:
566 *F. t. dacotiae* and *F .t. canariensis*) by means of contingency tables and exact
567 probabilities. Additionally, in an attempt to objectively condense the original
568 variable set of pathogens, we pooled them in groups with biological sense
569 according to their phylogeny and location of infections. We considered only
570 pathogens determined from blood by PCR in order to reach a largely enough
571 sample size with complete data for all individuals. Thus, we used the number of
572 bacterial (Phylum Actinobacteria, Proteobacteria), virus, and haematozoa
573 (protozoan from blood, Phylum Apicomplexa, Euglenozoa) genera, as well as

574 the presence or not of *Chlamydophila psittaci* (Phylum Chlamydiae),
575 *Mycoplasma* sp. (Division Firmicutes), the fungi *Candida albicans* (Phylum
576 Ascomycota) and *Trichomonas gallinae* (protozoan from the oral cavity, Phylum
577 Metamonada), infecting each individual kestrel. On this data set, we conducted
578 Categorical Principal Components Analysis (CatPCA) to obtain mutually
579 uncorrelated composite factors or dimensions [98]. Factor scores of the
580 individual birds on the resulting dimensions were extracted and used as
581 dependent variables of MANOVA to test for differences between
582 species/subspecies and age (nestlings and adults).
583

584 **Acknowledgments**

585 This study was funded by the Micinn (formerly MCyT) of the Spanish
586 Government (projects CGL2004-04120 and CGL2007-61395/BOS) and the
587 CSIC, which also provided research grants to M. Alcaide. We also thank J.C.
588 Illera for hepful comments and suggestions to an early draft of this manuscript.
589

590 **REFERENCES**

591

592 1. Sommer S (2005) The importante of immune gene variability in evolutionary
593 ecology and evolution. Front Zool 2: 16
594 2. Piertney SB, Oliver MK (2006) The evolutionary ecology of the major
595 histocompatibility complex. Heredity 96: 7–21.
596 3. Klein J (1986) Natural history of the major histocompatibility complex. Wiley,
597 New York
598 4, Hughes AL, Nei M (1992) Maintenance of MHC polymorphism. Nature 355:

599 402-403
600 5. Robinson J, Waller MJ, Parham P, de Groot N, Bontrop R, Kennedy LJ et al
601 (2003) IMGT/HLA and IMGT/MHC: sequence databases for the study of the
602 major histocompatibility complex. Nucleic Acids Res 31:311–314.
603 6. Clarke A, Gaston KJ (2006) Climate, energy and diversity. Proc R Soc Lond
604 B 273: 2257-2266.
605 7. Tella, J.L., Blanco, G., Forero, M.G., Gajón, A., Donázar, J.A., Hiraldo, F.
606 (1999). Habitat, world geographic distribution and embryonic development of
607 hosts explain the prevalence of avian hematozoa at small spatial and
608 phylogenetic scales. Proc Natl Acad Sci USA 96: 1785-1789.
609 8. Dobson AP, McCallum H (1997) The role of parasites in bird conservation.
610 Pp 155-173 in Clayton DH and Moore J (Eds). Host-Parasite Evolution.
611 General Principles and Avian Models. Oxford University Press, Oxford.
612 9. Hubálek Z (2004) An annotated checklist of pathogenic microorganisms
613 associated with migratory birds. J Wildl Dis 40: 639-659.
614 10. Tella JL (2002) The evolutionary transition to coloniality promotes higher
615 blood parasitism in birds. J Evol Biol 15: 32-4
616 11. Tella JL, Scheuerlein A, Ricklefs RE (2002). Is cell-mediated immunity
617 related to the evolution of life-history strategies in birds? Proc R Soc Lond B
618 269:1059-1066.
619 12. Friend M, Franson CJ (1999) Field Manual of Wildlife Diseases.
620 U.S.Goverment Printing Office and USGS, Washington, D.C.
621 13. Lumeij JT, Remple JD, Redig PT, Lierz M Cooper, JE. (2000) Raptor
622 Biomedicine III. Zoological Education Network. Lake Worth, Florida.
623 14. Hambuch TM, Lacey EA (2002) Enhanced selection for MHC diversity in

social tuco-tucos. Evolution 56: 841-845

15. Prugnolle F, Manica A, Charpentier M, Guegan JF, Guernier V, Balloux F (2005). Worldwide HLA class I diversity: human colonisation history and pathogen-driven selection. Curr Biol 15: 1022-1027.

16. Blais J, Rico, C, van Oosterhout, C, Cable J, Turner GF, Bernatchez, L (2007) MHC Adaptive Genetic Divergence between Closely Related and Sympatric African Cichlids. Plos One 8: E734, 1-12

17. Dionne M, Miller KM, Dodson JJ, Caron F, Bernatchez L (2007) Clinal variation in MHC diversity with temperature: evidence for the role of host-pathogen interaction on local adaptation in Atlantic Salmon. Evolution 61: 2154-2164.

18. Møller AP, Erritzøe J (1998) Host immune defence and migration in birds. Evol Ecol 12: 945-953.

19. Richardson D, Westerdahl H (2003) A comparison of MHC diversity in two Acrocephalus species: the outbred great reed warbler and the inbred Seyshelles warbler. Mol Ecol 12: 3523–3529.

20. Hansson B, Richardson DS (2005) Genetic variation in two endangered Acrocephalus species compared to a widespread congener: estimates based on functional and random loci. Anim Cons 8: 83-90.

21. Ekblom R, Sæther SA, Jacobson P, Fiske P, Sahlman T, Grahn M, Kålås JA, Höglund, J. (2007) Spatial patterns of MHC class II variation in the great snipe (Gallinago media). Mol Ecol 16: 1439-1451.

22. Alcaide M, Edwards SV, Negro JJ, Serrano D and Tella JL (2008) Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (Falco naumanni). Mol Ecol 17: 2652-2665.

649 23. Ekblom R, Saether SA, Grahn M, Fiske J, Kålås JA, Höglund J (2004).
650 Major histocompatibility complex variation and mate chioce in a lekking bird,
651 the great snipe (Gallinago media). Mol Ecol 13: 3821-3828.
652 24. Richardson DS, Komdeur J, Burke T, von Schantz T (2005) MHC-based
653 patterns of social and extra-pair mate choice in the Seychelles warbler. Proc
654 R Soc Lond B 272: 759–767.
655 25. Bonneaud C, Chaste O, Federici P, Westerdahl H and Sorci G (2006b)
656 Complex Mhc -based mate choice in a wild passerine. Proc R Soc Lond
657 B 273: 1111-1116.
658 26. Bonneaud C, Pérez-Tris J, Federici P, Chastel O, Sorci G (2006a) Mhc
659 alleles associated with local resistance to malaria in a passerine. Evolution
660 60: 383-389.
661 27. Loiseau C, Zoorob R, Garnier S, Birard J, Federici P, Julliard R, Sorci G
662 (2008) Antagonistic effects of a Mhc class I allele on malaria-infected house
663 sparrows. Ecol Lett 11: 258–265.
664 28. Groombridge JJ, Jones CG, Bayes MK, van Zyl AJ, Carrillo J, Nichols RA,
665 Bruford MW (2002). A molecular phylogeny of African kestrels with reference
666 to divergence across the Indian Ocean. Mol Phy Evol 25: 267-277.
667 29. Cramp S, Simmons KEL (eds) (1980). The Birds of the Western Palearctic,
668 Vol. 2. Oxford: Oxford University Press.
669 30. Clifford DL, Mazet JAK, Dubovi EJ, Garcelon DK, Coonan TJ, Conrad PA,
670 Munson L (2006) Pathogen exposure in endangered island fox (Urocyon
671 littoralis) populations: implications for conservation management. Biol Cons
672 131: 230-243.
673 31. Martí R, Del Moral JC (Eds.) (2003). Atlas de las Aves Reproductoras de

España. Dirección General de la Conservación de la Naturaleza-Sociedad Española de Ornitología, Madrid.

32. Ekblom R, Grahn M, Höglund J (2003) Patterns of polymorphism in the MHC class II of a non-passerine bird, the great snipe (Gallinago media). Immunogenetics 54: 734–741.

33. Alcaide M, Edwards SV, Cadahia SV and Negro JJ (2008) MHC class I genes of birds of prey: isolation, polymorphism and diversifying selection. Cons Genet.

34. Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? Trends Ecol Evol 21: 433-438.

35. Goüy de Bellocq J, Charbonell N, Morand S (2008) Coevolutionary relationship between helminth diversity and MHC class II polymorphism in rodents. J Evol Biol 21: 1144-1150.

36. Maré CJ, Graham DL (1973) Falcon Herpesvirus, the etiologic agent of inclusion body disease of falcons. Infect Immun 8: 118-125.

37. Palmgren H, Broman T, Waldenström J, Lindberg P, Aspán A, Olsen B (2004) Salmonella Amager, Campylobacter jejuni, and urease-positive thermophilic Campylobacter found in free-flying peregrine falcons (Falco peregrinus) in Sweden. J Wildl Dis 40: 583-587.

38. Schrenzel M, Oaks JL, Rotstein D, Maalouf G, Snook E, Sandfort C, Rideout B (2005) Characterization of a new species of adenovirus in falcons. J Clin Microbiol 43: 3402-3413.

39. Toro H, Hoerr FJ, Farmer K, Dykstra CC, Roberts SR, Perdue M (2005) Pigeon paramyxovirus: association with common avian pathogens in chickens and serologic survey in wild birds. Avian Dis 49: 92-98.

699 40. Tella JL, Forero MG, Donázar JA, Negro JJ, Hiraldo F (1997). Non-adaptive
700 adoptions of nestlings in the colonial lesser kestrel: proximate causes and
701 fitness consequences. Behav Ecol Sociobiol 40:253-260.
702 41. Forero MG, Tella JL, Donázar JA, Hiraldo F (1996). Can the interspecific
703 competition and nest site availability explain the decrease of lesser kestrel
704 populations?. Biol Conserv 78: 289-293.
705 42. Turcsányi I, Bistyák A, Matiz K, Kecskeméti S, Bölske G, Kiss I (2005)
706 Isolation of Mycoplasma columbinasale from pigeons in Hungary. Vet Rec
707 157: 235.
708 43. Rappole JH, Derrickson SR, Hubálek Z (2000) Migratory birds and spread
709 of West Nile virus in the Western Hemisphere. Emerg Infect Dis 6: 319-328.
710 44. Malkinson M, Banet C (2002) The role of birds in the ecology of West Nile
711 virus in Europe and Africa. Curr Top Microbiol Immunol 267: 309-322.
712 45. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, et
713 al.(2006) Global patterns of influenza a virus in wild birds. Science 312: 384–
714 388.
715 46. Munster VJ, Baas C, Lexmond P, Waldenstro¨m J, Wallensten A, et al.
716 (2007) Spatial, temporal, and species variation in prevalence of influenza A
717 viruses in wild migratory birds. PLoS Pathog 3: e61.
718 doi:10.1371/journal.ppat.0030061
719 47. López G, Jiménez-Clavero MA, Tejedor CG, Soriguer R, Figuerola J (2008)
720 Prevalence of West Nile Virus neutralizing antibodies in Spain is related to
721 the behavior of migratory birds. Vector Borne Zoonotic Dis. Doi:
722 10.1089/vbz.2007.0200
723 48. Alcaide M, Serrano D, Negro JJ, Tella JL, Laaksonen T, Müller C, Gal A,

724 Korpimäki E (2008) Population fragmentation leads to isolation by distance
725 but not genetic impoverishment in the philopatric Lesser Kestrel: a
726 comparison with the widespread and sympatric Eurasian Kestrel. Heredity.
727 49. Aguilar A, Roemer G, Debenham S, Binns M, Garcelon D, Wayne RK
728 (2004) High MHC diversity maintained by balancing selection in an otherwise
729 genetically monomorphic mammal. Proc Natl Acad Sci USA 101: 3490-3494.
730 50. Jarvi SI, Tarr CL, McIntosh CE, Atkinson CT, Fleischer RC (2004) Natural
731 selection of the major histocompatibility complex (Mhc) in Hawaiian
732 honeycreepers (Drepanidinae). Mol Ecol 13: 2157– 2168.
733 51. Crowl TA, Crist TO, Parmenter RR, Belowvsky G, Lugo AE (2008) The
734 spread of invasive species and infectious disease as drivers of ecosystem
735 change. Front Ecol Environ 6: 238-246.
736 52. Smith KF, Sax DF, Lafferty KD (2006) Evidence for the Role of Infectious
737 Disease in Species Extinction and Endangerment. Conserv Biol 20:1349-
738 1357.
739 53. Daszak P,Cunningham AA, Hyatt AD (2000) Emerging Infectious Diseases
740 of Wildlife-Threats to Biodiversity and Human Health. Science 287: 443-449.
741 54. Tella JL, Carrete M (2008). Broadening the role of parasites in biological
742 invasions. Front Ecol Environ 6: 11-12.
743 55. Van Riper C, Van RSG, Goff ML, Laird M (1986). The epizootiology and
744 ecological significance of malaria in Hawaiian USA land birds. Ecol Monog
745 56: 327–344.
746 56. Wikelski M, Foufopoulos J, Vargas H, Snell H (2004). Galápagos Birds and
747 Diseases: Invasive Pathogens as Threats for Island Species. Ecology and
748 Society 9(1): 5. [online] URL: http://www.ecologyandsociety.org/vol9/iss1/art5

749 57. Carrete M, Serrano D, López G, Illera JC, Vögeli M, Delgado A, Tella JL
750 (2008) Goats, birds, and emergent diseases: apparent and hidden effects of
751 exotic species on an island environment. Ecol Appl.
752 58. BirdLife International (2007) Species factsheets. Downloaded from
753 http://www.birdlife.org on
754 59. Madroño A, González C and Atienza JC (2004) Libro Rojo de las Aves de
755 España. Dirección General para la Biodiversidad –SEO/BirdLife. Madrid.
756 60. Alcaide M, Negro JJ, Serrano D, Tella JL, Rodriguez C (2005) Extra-pair
757 paternity in the Lesser Kestrel Falco naumanni: a re-evaluation using
758 microsatellite markers. Ibis 147: 608-611.
759 61. Nesje M, Roed KH, Lifjeld JT, Lindberg P, and Steens OF (2000) Genetic
760 relationship in the Peregrine Falcon (Falco peregrinus) analysed by
761 microsatellite DNA markers. Mol Ecol 9: 53–60.
762 62. Alcaide M, Edwards SV and Negro JJ (2007) Characterization,
763 Polymorphism and Evolution of MHC class II B genes in birds of prey. J Mol
764 Evol 65: 541-554.
765 63. Aparicio JM, Ortego J, Cordero PJ (2006) What should we weigh to
766 estimate heterozygosity, alleles or loci?. Mol Ecol 15: 4659–4665.
767 64. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor
768 and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser 41: 95-
769 98.
770 65. Bradley RD, Hillis DM (1997) Recombinant DNA sequences generated by
771 PCR amplification. Mol Biol Evol 14: 592-593.
772 66. Rozas J, Sánchez-DelBarrio JC, Messeguer X and Rozas R (2003) DnaSP,
773 DNA polymorphism analyses by the coalescent and other methods.

774 Bioinformatics 19: 2496-2497.
775 67. Rodi DJ, Mandava S, Makowski L (2004) DIVAA: analysis of amino acid
776 diversity in multiple aligned protein sequences. Bioinformatics 20:3481–3489.
777 68. Tella JL, Forero MG, Gajón A, Hiraldo F, Donázar JA (1996) Absence of
778 blood parasitization effects on Lesser Kesstrel fitness. Auk 113:253-256.
779 69. Bize P, Roulin A, Tella JL, Bersier L-F, Richner H (2004). Additive effects of
780 ectoparasites over reproductive attempts in the long-lived alpine swift. J Anim
781 Ecol 73: 1080-1088.
782 70. Tella JL, Forero MG, Donázar JA, Negro JJ, Hiraldo F (1997). Non-adaptive
783 adoptions of nestlings in the colonial lesser kestrel: proximate causes and
784 fitness consequences. Behav Ecol Sociobiol 40: 253-260.
785 71. Blanco G, Tella JL, Potti J, Baz A (2001). Feather mites in birds: costs of
786 parasitism or conditional outcomes? J Avian Biol 32: 271-275.
787 72. Holmes AR, Cannon RD, Sheperd MG, Jenkinson HF. (1994) Detection of
788 Candida albicans and other yeast in blood by PCR. J Clin Microbiol 32: 228-
789 231.
790 73. Blanco G, Lemus JA, Grande J, Gangoso L, Grande JM, Donázar JA,
791 Arroyo B, Frías O and Hiraldo F (2007) Geographical variation in cloacal
792 microflora and bacterial antibiotic resistance in a threatened avian scavenger
793 in relation to diet and livestock farming practices. Environ Microbiol 9: 1738-
794 1749.
795 74. Aranaz A, Liébana E, Mateos A, Domínguez L (1997) Laboratory Diagnosis
796 of Avian Mycobacteriosis. Semin Avian Exot Pet Med 6: 9-17.
797 75. Khan AA, Cerniglia,CE. (1994) Detection of Pseudomonas aeruginosa from
798 clinical and environmental samples by amplification of the exotoxin A gene

using PCR. Appl Environ Microbiol 60: 3739-3745.

76. Saxena MK, Singh VP, Kumar AA, Chaudhuri P, Pal Singh V,Shivachandra, SB, Biswas A, Sharma, B. (2006) REP-PCR Analysis of Pasterella multocida from wild and domestic animals in India. Vet Res Com 11259-006-3321-5.

77. Petersen L, Newell DG (2001) The ability of Fla-typing schemes to discriminate between strains of Campylobacter jejuni. J Appl Microbiol 91: 217–224.

78. Schettler E, Fickel J, Hotzel H, Sachse K, Streich WJ, Wittstatt U, Frolich K (2003) Newcastle disease virus and Chlamydia psittaci in free-living raptors from eastern Germany. J Wildlife Dis 39: 57–63.

79. Mekkes DR, Feberwee A (2005) Real-time polymerase chain reaction for the qualitative and quantitative detection of Mycoplasma gallisepticum. Avian Path 34: 348–354.

80. Tadese T, Reed WM (2003) Use of restriction fragment length polymorphism, immunoblotting, and polymerase chain reaction in the differentiation of avian poxviruses. J Vet Diag Invest 15: 141–50.

81. Cardoso M, Hyatt A, Selleck P, Lowther S, Prakash V, Pain D, Cunningham AA Boyle , D (2005) Phylogenetic analysis of the DNA polymerase gene of a novel alphaherpesvirus isolated from an Indian Gyps vulture. Virus Genes 30: 371-381.

82. Hsu CM, Ko CY, Tsaia HJ (2006) Detection and sequence analysis of avian polyomavirus and psittacine beak and feather disease virus from psittacine birds in Taiwan. Avian Dis 50: 348-353.

83. Kiss I, German P, Sami L, Antal M, Farkas T, Kardos G, Kecskemeti S, Dan A, Belak S (2006) Application of real-time RT-PCR utilising lux (light upon

824 extension) fluorogenic primer for the rapid detection of avian influenza
825 viruses. Acta Vet Hung 54: 525–33.
826 84. Zhang Y, Liu M, Shuidong O, Hu QL, Guo DC, Chen HY, Han Z (2006)
827 Detection and identification of avian, duck, and goose reoviruses by RT-PCR:
828 goose and duck reoviruses are part of the same genogroup in the genus
829 Orthoreovirus. Arch Virol 151: 1525–1538.
830 85. Farkas T, Antal M, Sami L, German P, Kecskemeti S, Kardos G, Belak S,
831 Kiss I (2007) Rapid and simultaneous detection of avian influenza and
832 Newcastle disease viruses by duplex polymerase chain reaction assay.
833 Zoonose Public Health 54: 38–43.
834 86. Schrenzel M, Snook E, Gagneux P. (2007). Molecular assays for detection
835 of falcon adenovirus.J Vet Diagn Invest. 2007: 19:479-485.
836 87. Hofle U, Blanco JM, Crespo E et al. (2007) West Nile virus in the
837 endangered Spanish imperial eagle, Vet. Microbiol 129: 171-178
838 88. Potti J, Blanco G, Lemus JA, Canal D (2007) Infectious offspring: how birds
839 acquire and transmit an avian polyomavirus in the wild. PloS ONE 2(12):
840 e1276. doi:10.1371/journal.pone.0001276.
841 89. Hellgren O, Waldenström J, Bensch S (2004) A new PCR assay for
842 simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus
843 from avian blood. J Parasitol 90: 797-802.
844 90. Stone RG, Montiel-Parra G, Perez TM (2005) A survey of selected parasitic
845 and viral pathogens in four species of Mexican parrots, Amazona autumnalis,
846 Amazona oratrix, Amazona viridigenalis, and Rhynchopsitta pachyrhyncha. J
847 Zoo Wildl Med 36: 245-249.
848 91. Cosgrove CL, Day KP, Sheldon BC (2006). Coamplification of

849 Leucocytozoon by PCR diagnostic tests for avian malaria: a cautionary note.
850 J Parasitol. 92:1362-1365.
851 92. Valkiŭnas G, Bensch S, Iezhova TA, Krizanauskiené A, Hellgren O,
852 Bolshakov CV (2006). Nested cytochrome b polymerase chain reaction
853 diagnostics underestimate mixed infections of avian blood haemosporidian
854 parasites: microscopy is still essential. J Parasitol. 92: 418-422.
855 93. Grabensteiner E, Hess M (2006) PCR for the identification and
856 differentiation of Histomonas meleagridis, Tetratrichomonas gallinarum and
857 Blastocystis spp. Vet Parasitol 142: 223-230.
858 94. Forbes NA, Fox MT (2005) Field trial of a Caryospora vaccine for controlling
859 clinical coccidiosis of falcons .Vet Rec. 156: 134-128.
860 95. Greiner EC, Ritchie BW (1994) Parasites. In: Avian Medicine: principles and
861 applications. Ritchie, BW; Harrison, G and Harrison, LR (eds). Wingers
862 Publishing Inc, Lake Worth, Florida. Pp: 1007-1029.
863 96. Clyde VL, Patton S (2001) Parasitism of caged birds. In: Manual of Avian
864 Medicine. Olsen GH & Orosz, SE (eds). Mosby. Pp 424-448.
865 97. Jovani R, Tella JL (2006) Parasite prevalence and sample size:
866 misconceptions and solutions. Trends Parasitol 22:214-218.
867 98. Meulman JJ, Van der Kooij AJ, Heiser WJ (2004). Principal components
868 analysis with nonlinear optimal scaling transformations for ordinal and
869 nominal data. In D. Kaplan (Ed.) Handbook of quantitative methodology for
870 the social sciences (pp. 49-70). Thousand Oaks, CA: Sage Publications.
871 99. Møller AP, Erritzoe J (1996) Parasite virulence and host immune defence:
872 Host immune response is related to nest re-use in birds.
873 Evolution 50: 2066-2072.

100. Møller AP, Merino S, Brown CR & Robertson RJ (2001) Immune defense and host sociality: a comparative study of swallows and martins. Am Nat 158: 136–145.

TABLES

Table 1. Hypotheses posed to explain variability in pathogens between host species with different ecology and life histories, and predictions on the resulting pathogen burdens in our study model. LK: lesser kestrel, CEK: continental Eurasian kestrel, IEK: island Eurasian kestrel

Hypothesis	Predictions	References
Nest reuse in cavity nesters	LK = CEK	[99]
Body size	LK = CEK	[11]
Development period	LK = CEK	[7], [11]
Longevity	LK = CEK	[11]
Migratory behaviour	LK > CEK	[9]
Colonial breeding	LK > CEK	[100], [10]
Trophic diversity	LK < CEK	[12]
Geographic range	LK < CEK	[7]
Habitat generalist	LK < CEK	[8]
Aridity	LK < CEK	[7]
Insularity	IEK < CEK	[8]

935 **Table 2**. Diversity of pathogens (Shannon-Wiener index), richness (number of
936 pathogen species), prevalence (percentage of individuals infected by at least
937 one pathogen), and individual richness (mean number of pathogens per
938 individual host) of pathogens infecting the different species, subspecies and age
939 classes of kestrels sampled. Only pathogens determined from blood by PCR
940 were considered in order to analyze complete data for all individuals.

941

	Adult Birds				Nestlings	
	Falco t. tinnunculus	***Falco t. dacotiae***	***Falco t. canariensis***	***Falco naumanni***	***Falco t. tinnunculus***	***Falco naumanni***
Diversity	3.37	1.97	2.28	2.58	1.98	1.88
Richness	26	16	14	19	23	20
Prevalence	100	94.1	90.0	97.8	86.9	89.1
Individual richness	4.05	2.59	2.10	3.69	1.96	1.91

942
943
944
945
946

Table 3. Results from the CatPCA indicating the correlation (component loading coefficient, major contributors in bold) between groups of pathogens and the resulting four dimensions (D). The variance explained by each pathogen group is shown in brackets.

Pathogens	D1	D2	D3	D4	% variance
Viruses	**0.664 (0.441)**	-0.044 (0.002)	-0.262 (0.068)	-0.204 (0.042)	0.553
Bacteria	**0.665 (0.442)**	-0.149 (0.022)	-0.205 (0.042)	0.266 (0.071)	0.577
Haematozoa	0.253 (0.064)	**0.644 (0.415)**	-0.003 (0.000)	-0.532 (0.283)	0.762
Mycoplasma	0.073 (0.005)	0.396 (0.157)	0.512 (0.262)	**0.601 (0.362)**	0.786
C. psitacci	0.496 (0.246)	-0.362 (0.131)	0.176 (0.031)	0.182 (0.033)	0.441
T. gallinae	0.120 (0.014)	**0.591 (0.349)**	-0.446 (0.199)	0.393 (0.154)	0.717
C. albicans	0.359 (0.129)	0.126 (0.016)	**0.668 (0.446)**	-0.247 (0.061)	0.652
Eigenvalue	1.342	1.092	1.049	1.005	4.488
% variance	19.168	15.600	14.985	14.363	64.116

Table 4. Results from MANOVAs testing differences between species/subspecies and age classes in the four dimensions derived from a categorical PCA that condense variation in the pathogen community. Significant results are in bold. For the comparison of subspecies only adult individuals were included because nestlings of the insular subspecies were not sampled.

	Species/subspecies		Age		Species x Age	
Dimension	***F***	***P***	***F***	***P***	***F***	***P***
Species						
D1	9.272	**0.002**	123.544	**<0.0001**	0.945	0.331
D2	4.060	**0.044**	3.450	0.064	0.026	0.873
D3	.206	0.650	3.606	0.058	7.013	**0.008**
D4	2.543	0.111	1.093	0.296	8.853	**0.003**
Subspecies						
D1	22.429	**<0.0001**				
D2	1.466	0.230				
D3	0.412	0.523				
D4	12.476	**0.001**				

977 **Table 5**. Polymorphism statistics at 8 microsatellites and two MHC loci in
978 kestrels. We show the number of alleles at microsatellites and MHC loci as well
979 as average estimates of homozygosity by loci (H_L). Polymorphism statistics at
980 MHC sequences include the number of segregating sites S), total number of
981 mutations (η), nucleotide diversity (π) and the average number of nucleotide
982 differences between unique alleles (k). LK: lesser kestrel (N=25), CEK:
983 continental Eurasian kestrel (N=25), IEK: island Eurasian kestrel (N=25).
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998

999

1000

	Microsatellites		MHC Class II						MHC Class I					
	Alleles	H_L	Alleles	H_L	S	η	π	K	Alleles	H_L	S	η	π	K
LK	61	0.172	31	0	61	74	0.086	22.68	18	0.08	39	41	0.033	9.15
CEK	58	0.162	41	0	72	89	0.090	24.31	23	0.04	33	38	0.039	10.99
IEK	44	0.260	10	0.2	56	67	0.095	25.78	6	0.28	17	18	0.031	8.45

FIGURE LEGENDS

Figure 1. Differences between species/subspecies and age classes in the four dimensions derived from a categorical PCA that condense variation in the pathogen community. Black dots represent fledglings and open dots adults. Sample sizes are shown in the plot for D2.

Figure 2. Amino acid diversity (d) at the putative PBR [22,33] of class I and class II loci in mainland populations of Eurasian and the lesser kestrels.

Figure 3. Sampled locations of the Eurasian kestrel (black asterisks) and the lesser kestrel (white asterisks) for genetic analyses. The origin of the individuals sampled for pathogen surveys is indicated by dark grey areas.

FIG 1.

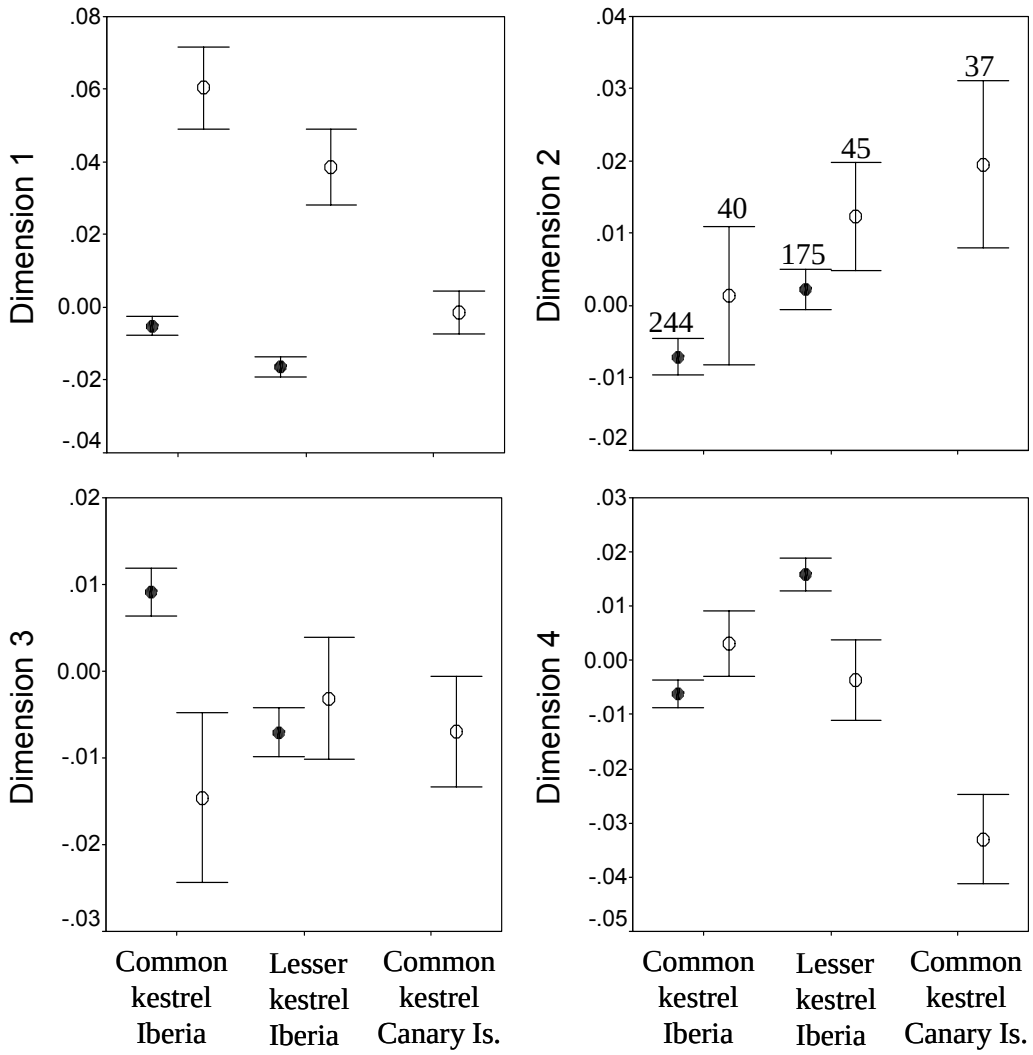
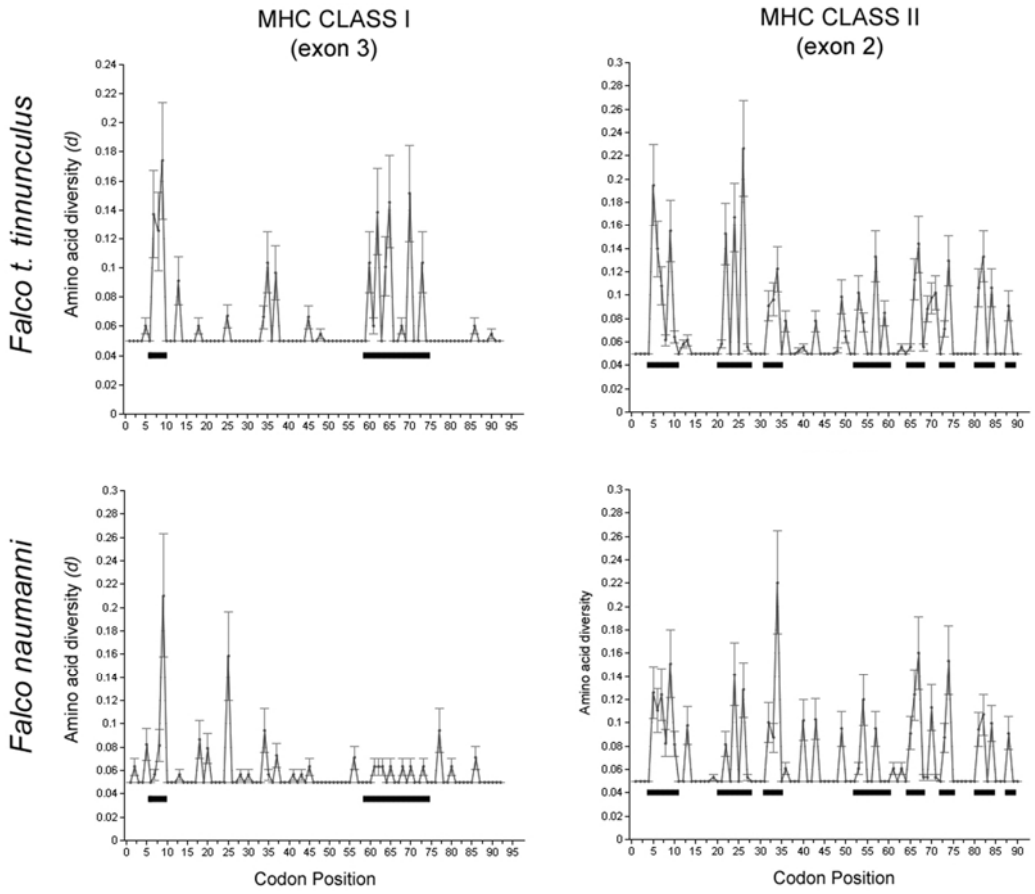
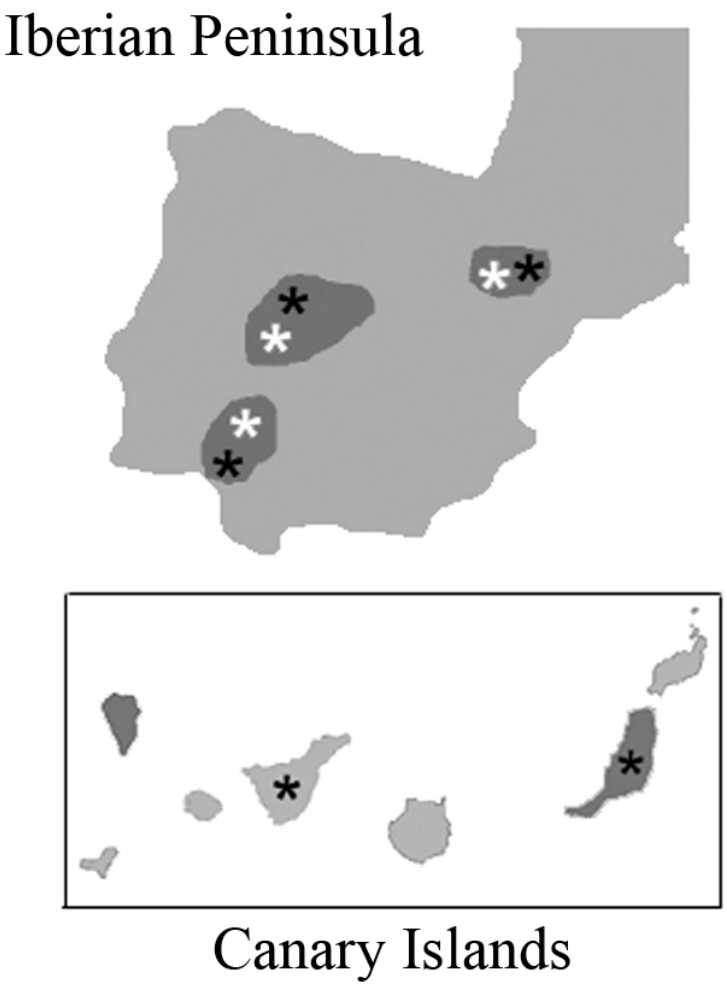

Dimension 1
.08
.06
.04
.02
0.00
-.02
-.04
Dimension 2
.04
.03
.02
.01
0.00
-.01
-.02
244
40
175
45
37
Dimension 3
.02
.01
0.00
-.01
-.02
-.03
Dimension 4
.03
.02
.01
0.00
-.01
-.02
-.03
-.04
-.05
Common kestrel Iberia
Lesser kestrel Iberia
Common kestrel Canary Is.
Common kestrel Iberia
Lesser kestrel Iberia
Common kestrel Canary Is.

FIG 2.


MHC CLASS I
(exon 3)
MHC CLASS II
(exon 2)
Falco t. tinnunculus
Falco naumanni
Amino acid diversity (d)
Amino acid diversity
Codon Position
Codon Position

1050 **FIG 3.**

1051



1052

1053

1054

1055

1056

SUPPORTING INFORMATION

Table S1. Ecological profiles and life history traits of the Eurasian kestrel (*Falco tinnunculus*) and the lesser kestrel (*Falco naumanni*). See [29] for details.

	Falco tinnunculus	***Falco naumanni***
Body mass	140 – 300 g	100 – 200 g
Clutch size	3 – 6 eggs	3 – 5 eggs
Incubation period	27 – 29 days	28 – 29 days
Nestling period	27 – 32 days	28 days
Life span	16 yr	13 yr
Niche amplitude		
Altitudes	0-5000 m	0-2750 m
Habitats	Wide tolerance - Generalist (steppes and pseudosteppes, semi-deserts, low dense forests, urban environments)	Specialist Steppes and pseudosteppes, urban environments
Nests	Mostly cavity-nester, using cliffs, human structures and trees . Also in corvid nests and exceptionally on the ground	Cavity-nester, using mostly human structures, exceptionally in cliffs, on the ground or in tree holes
Distributional range in the Western Paleartic Resident (black areas) Migrant breeding (grey areas)		
	Breeding latitudes: up to 70ºN	Breeding latitudes : 30-50º N

Breeding system	Usually solitary breeder	Mostly colonial
Migratory status	Sedentary (i.e. Canary Islands) , partially sedentary (i.e. Iberian Peninsula) or short-medium distance migrator (i.e. North Europe)	Trans-Saharian, long-distance migrator
Diet	Euriphagous (small mammals, birds, reptiles and insects)	Estenophagous (Insect specialist)

Table S2. Prevalence (percentage of individuals infected) of 35 avian pathogens in lesser (*Falco naumanni*) and Eurasian kestrels (*Falco tinnunculus*). Results are separated for each age class and subspecies. Sample sizes are given in bold.

	PREVALENCE					
	Adult Birds				Nestlings	
	Falco t. tinnunculus	***Falco t. dacotiae***	***Falco t. canariensis***	***Falco naumanni***	***Falco t. tinnunculus***	***Falco naumanni***
Fungi	***n* = 40**	***n* = 17**	***n* = 20**	***n* = 45**	***n* = 244**	***n* = 175**
Candida albicans	25.0	23.5	15.0	33.3	25.4	4.0
Bacteria	***n* = 40**	***n* = 17**	***n* = 20**	***n* = 45**	***n* = 244**	***n* = 175**
Campylobacter sp.	10.0	0.0	0.0	22.2	7.0	1.1
E. coli enterotoxigenic strain	30.0	17.6	5.0	6.7	11.9	21.1
Mycobacterium avium	2.5	0.0	0.0	0.0	0.0	0.0
Pasterella multocida	17.5	0.0	0.0	0.0	1.2	1.1
Pseudomonas aeruginosa	5.0	0.0	0.0	0.0	2.0	0.0
Salmonella sp.	22.5	17.6	30.0	20.0	5.3	2.9
Chlamydophila psittaci	52.5	11.8	15.0	37.8	34.0	26.3
Mycoplasma sp.	40.0	23.5	20.0	44.4	32.4	46.9
Viruses	***n* = 40**	***n* = 17**	***n* = 20**	***n* = 45**	***n* = 244**	***n* = 175**
Adenovirus	35.0	0.0	0.0	26.7	7.0	1.7

Circovirus	0.0	0.0	0.0	0.0	0.0	0.0
Herpesvirus	7.5	0.0	0.0	0.0	0.0	0.0
Influenzavirus (IH5)	0.0	0.0	0.0	0.0	0.0	0.0
Influenzavirus (IH7)	12.5	0.0	0.0	8.9	1.2	9.1
Influenzavirus (IH9)	0.0	0.0	0.0	0.0	0.0	0.0
Paramixovirus	40.0	0.0	0.0	8.9	7.8	11.4
Polyomavirus	0.0	0.0	0.0	0.0	0.0	0.0
Poxvirus	12.5	29.4	25.0	24.4	7.0	10.9
Reovirus	5.0	0.0	0.0	20.0	8.2	1.1
West Nile virus	25.0	58.8	40.0	53.3	16.4	13.1
Hemoparasites	***n* = 40**	***n* = 17**	***n* = 20**	***n* = 45**	***n* = 244**	***n* = 175**
Haemoproteus sp.	0.0	5.9	0.0	0.0	0.0	0.0
Leucocytozoon sp.	22.5	35.3	21.4	20.0	10.2	4.6
Plasmodium sp.	0.0	11.8	15.0	0.0	0.0	0.0
Trypanosoma sp.	0.0	5.9	5.0	0.0	0.0	0.0
Trichomonas	***n* = 40**	***n* = 17**	***n* = 20**	***n* = 45**	***n* = 244**	***n* = 175**
Trichomonas gallinae	40.0	17.6	25.0	42.2	19.3	35.4
Intestinal parasites	***n* = 40**	***n* = 4**	***n* = 4**	***n* = 25**	***n* = 100**	***n* = 102**
Coccidia						
Caryospora sp.	42.5	50.0	0.0	44.0	11.0	10.8
Eimeria sp.	22.5	0.0	0.0	28.0	4.0	3.9

Isospora sp.	17.5	0.0	0.0	0.0	0.0	0.0
Cestodes						
Cladotaenia sp.	0.0	0.0	0.0	20.0	10.0	8.8
Unciunia sp.	0.0	50.0	75.0	0.0	0.0	0.0
Nematodes						
Serratospiculum sp.	0.0	0.0	0.0	0.0	0.0	0.0
Porrocaecum sp.	15.0	0.0	0.0	56.0	9.0	9.8
Ascaridia sp.	47.5	50.0	0.0	0.0	4.0	0.0
Capillaria sp.	27.5	0.0	25.0	80.0	2.0	18.6
Cyrnea sp.	30.0	25.0	25.0	0.0	5.0	5.9

Table S3: Statistical significance of the differences in prevalence of particular pathogens between continental Eurasian and Lesser kestrels (considering nestlings and adults separately), as well as between continental and insular adult Eurasian kestrels (pooling both island subspecies) by means of contingency tables and exact probabilities. Significant results are in bold.

Pathogen	Comparison				
	nestling/adult lesser kestrels	**nestling/adult Eurasian kestrels**	**Eurasian/lesser kestrels adults**	**Eurasian/lesser kestrels nestlings**	**Insular/continental Eurasian kestrels Adults**
Fungi					
Candida albicans	**<0.0001**	1.000	0.478	**<0.0001**	0.590
Bacteria					
Campylobacter sp.	0.141	0.513	0.153	**0.007**	0.116
E. coli (enterotoxigenic)	**0.029**	**0.005**	**0.009**	**0.014**	**0.050**
Mycobacterium avium	-	0.141	0.471	-	1.000
Pasterella multocida	0.673	**<0.0001**	**0.004**	1.000	**0.012**
Pseudomonas aeruginosa	-	0.257	0.218	0.078	0.494
Salmonella sp	**<0.0001**	**<0.0001**	0.797	0.236	1.000
Chlamydophila psitacci	0.141	**0.033**	0.196	0.107	**0.001**
Mycoplama sp.	0.867	0.369	0.826	**0.003**	0.092
Virases					
Adenovirus	**<0.0001**	**<0.0001**	0.482	**0.018**	**<0.0001**
Circovirus	-	-	-	-	-
Herpesvirus	-	**0.003**	1.000	-	0.241
Influenzavirus IH5	-	-	-	-	-
Influenzavirus IH7	1.000	**0.002**	0.729	**<0.0001**	0.055
Influenzavirus IH9	-	-	-	-	-

Paramixovirus	0.791	**<0.0001**	**<0.0001**	0.234	**<0.0001**
Polyomavirus	-	-	-	-	-
Poxvirus	**0.027**	0.333	0.178	0.215	0.151
Reovirus	**<0.0001**	0.556	0.053	**<0.001**	0.494
West-Nile virus	**<0.0001**	0.261	**0.014**	0.407	**0.036**
Haemoparasites					
Haemoproteus sp.	-	-	-	-	0.493
Leucocytozoon sp.	**0.002**	**0.036**	0.797	**0.042**	0.589
Plasmodium sp.	-	-	-	-	**0.025**
Trypanosoma sp.	-	-	-	-	0.228
Tricomonas					
Trichomonas gallinae	0.488	**0.005**	1.000	**<0.0001**	0.092
Intestinal parasites					
Coccidians					
Caryospora sp.	**<0.001**	**<0.0001**	1.000	1.000	-
Eimeria sp.	**<0.001**	**0.002**	0.765	1.000	0.322
Isospora sp.	-	**<0.0001**	**0.038**	-	0.333
Cestodes					
Cladotenia sp.	0.149	0.062	**0.006**	0.814	**<0.0001**
Unciunia sp.	-	-	-	-	-
Nematodes					
Serratospiculum sp.	-	-	-	-	-
Porrocaecum sp.	**<0.0001**	0.365	**<0.001**	1.000	0.571
Ascaridia sp.	-	**<0.0001**	**<0.0001**	0.058	0.432
Capillaria sp.	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.659
Cyrnea sp.	0.351	**<0.0001**	**0.002**	1.000	1.000

1134
1135
1136
1137
1138
1139
1140
1141

Figure S1. Putative amino acid sequences of an MHC class I locus (exon 3) in different species and subspecies of kestrels. LK: lesser kestrel, CEK: continental Eurasian kestrel, IEK: island Eurasian kestrel.

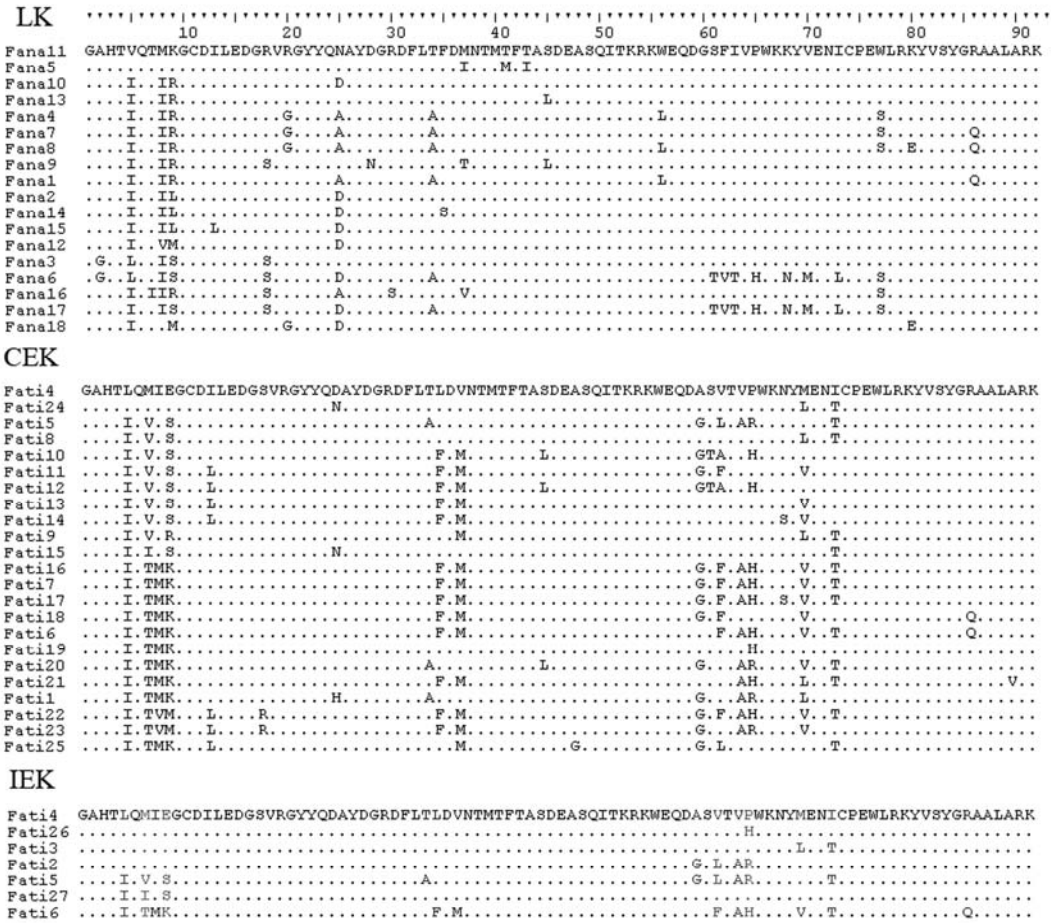


Figure S2. Putative amino acid sequences of an MHC class IIB locus (exon 2) in different species and subspecies of kestrels. LK: lesser kestrel, CEK: continental Eurasian kestrel, IEK: island Eurasian kestrel.