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Genetic differences between early- and late-breeding Eurasian kestrels

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ABSTRACT

Question: Is there a genetic difference between early-breeding kestrels (*Falco tinnunculus*), which typically lay five or six eggs, and late-breeding kestrels, which, although sympatric, usually lay only four eggs?

Field site: Cultivated and set-aside fields of a Mediterranean area of 1200 km^2, near Rome, Italy.

Methods: We compared the genotypes of chicks belonging to the two groups by analysing the difference at five microsatellite loci.

Conclusions: Early- and late-hatched chicks are genetically dissimilar, with an average F_{ST}-value of 0.058. This result suggests assortative mating-by-time and the possibility of adaptive divergence between early and late breeders within sympatric populations.

Keywords: clutch size, Eurasian kestrel, genetic differentiation, laying date, microsatellites, population genetics, timing of breeding.

INTRODUCTION

The time of breeding (i.e. egg laying) in birds is a primary factor affecting reproductive success, and breeding generally peaks when food conditions are optimal for chick rearing (Lack, 1954). For birds breeding at high latitudes, the laying period in general is relatively synchronous, but for some species can spread over weeks or months at a given location. To date, a number of proximate factors have been observed to influence the beginning of egg laying in birds (Svensson, 1995, 1997; Lambrechts *et al.*, 1997). For example, the onset of laying for the same female can vary from one year to another depending on her age (Blondel *et al.*, 1990), physiological conditions (Hasselquist *et al.*, 2001), or breeding experience (Grieco *et al.*, 2002). On the other hand, it has been suggested that the beginning of egg laying can sometimes be

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constant and heritable (Van Noordwijk *et al.*, 1981; for a review, see Hendry and Day, 2005). It therefore remains unclear the extent to which variation among birds in laying date depends on flexible decisions versus genetic programming.

The time of egg laying in birds often covaries with phenotypic traits, and the strongest pattern is that later clutches are of smaller size (Perrins, 1970; Lepage *et al.*, 2000; Winkler *et al.*, 2002). This seasonal decline could be the result of decreased food supply, harsher weather conditions, or reduced parental care (Hansson *et al.*, 2000). Another possibility is that only good-quality individuals (e.g. in good condition or with good foraging skills) can breed early and have large clutches. Low-quality individuals perhaps must instead breed late and lay fewer eggs (Verhulst *et al.*, 1995). Many authors thus deem the seasonal decline in clutch size to be a result of plasticity depending on individual condition. An untested alternative, however, is that breeding date is genetically determined, thus reducing gene flow between early- and late-breeding groups and allowing adaptive genetic divergence in clutch size (Hendry and Day, 2005). In this paper, we examine a part of this alternative hypothesis by testing whether gene flow is limited between early and late breeders, thus providing the potential for temporal clines in selection to generate adaptive phenotypic clines.

We addressed this question in a population of kestrels in central Italy, where the onset of egg laying typically spans a period of 65 days. Some inter-annual variation is also present, such that the breeding seasons observed in 2003 and 2004 were limited to 55 days (Fig. 1). Within this population, birds breeding at the beginning of the season typically lay five or six eggs, whereas those breeding late in the season typically lay only four eggs (Fig. 2). Kestrels are known to show assortative mating based on body size (Hakkarainen *et al.*, 1996) and colour (Palokangas *et al.*, 1994). We hypothesized that they might also show assortative mating by breeding time, and that we could detect the genetic signatures of this tendency. If present, assortative mating-by-time could help maintain the variation in laying dates in this population, and perhaps facilitate the formation of partial reproductive isolation between early and late breeders in sympatry. We specifically examined genetic differences at neutral markers between individuals at the extremes of the breeding time distribution in this Mediterranean kestrel population.

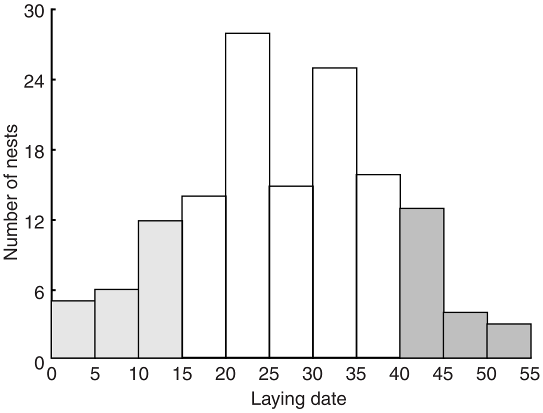

Fig. 1. Distribution of laying dates recorded at 141 nests of Eurasian kestrel (*Falco tinnunculus*) during 2003 and 2004. Laying date is standardized to the mean date of each season. This study examined genetic differentiation between early breeders (pale grey columns on the left-hand side) and late breeders (dark grey columns on the right-hand side).

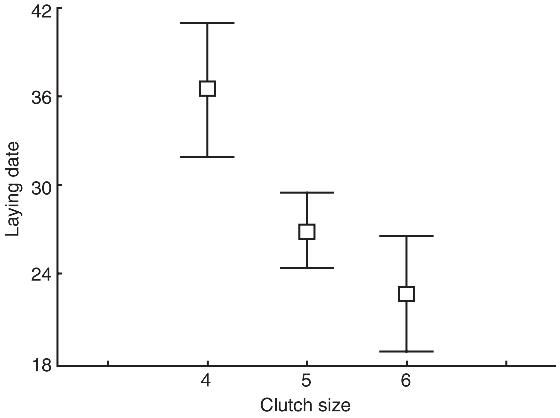

Fig. 2. Clutch sizes of Eurasian kestrels (*Falco tinnunculus*) in relation to laying date (mean ± standard error). Clutches of five or six eggs are typical for early breeders, whereas clutch sizes of four eggs are typical for late breeders (one-way ANOVA: $F_{1,138} = 10.69$, $P < 0.001$; normality was checked using the Kolmogorov-Smirnoff test). *Post-hoc* tests showed that clutches of four eggs were laid later than clutches of five eggs (9.41 ± 2.62, $P = 0.01$) and six eggs (13.64 ± 2.97, $P = 0.0001$), whereas no differences were evident between the dates of laying for five- versus six-egg clutches (4.24 ± 2.19, $P = 0.164$).

MATERIALS AND METHODS

Samples were collected during 2003 and 2004 over 1200 km^2 near Rome (central Italy), an area characterized mainly by cultivated and set-aside fields. The kestrel chicks we sampled had hatched in nest boxes on power lines or other man-made structures. Starting in March, we visited nest boxes regularly to assess occupation, onset of laying, and clutch size. Hatching dates for individual nestlings were determined by visits to the nests in the peri-hatching period or by a growth curve (error: ± 1 day; unpublished data). Since the laying date of the first clutches in our study population varied between years, we standardized individual laying date in relation to the mean date in a given year. We then considered early clutches to be those laid during the first 15 days of the reproductive season and late clutches to be those laid during the last 15 days. There was an approximately one-month period between the latest early clutch and the earliest late clutch we analysed.

We compared DNA samples collected from 20 early broods with those collected from 20 late broods. The samples were collected from nests located in different places throughout the 1200 km^2 study area. Specifically, fourteen nests were collected in the north-east of Rome, ten in the north-west, five in the west, six in the south-east, and five in the south-west. Within each area, most nests were 1 to 10 km distant from each other. Early and late nests were sampled in each area and so the timing differences were not the result of spatial differences.

Given the fidelity of kestrels to their nests (Village, 1990; personal observation), and the fact that the 40 DNA samples were collected from different nests, we are confident that none of the offspring analysed had the same pair of parents. When chicks were 9–31 days old, blood samples were taken from the brachial vein and 20–50 μl blood were spotted onto filter paper (number 903; Schleicher and Shuell, Dassel, Germany) previously treated with $UV_{\lambda 256nm}$. The spotted blood was allowed to air dry for 1 h and the filter was then placed in a sealed plastic bag. Only one chick per brood was used. The DNA samples were prepared by cutting 2 mm^2 of the blood-spotted paper using the QIAGEN-QIAamp DNA Blood mini kit, following the manufacturer's instructions. Purified, alcohol-precipitated DNA was resuspended in water in a final volume of 20 μl.

We analysed six polymorphic microsatellite loci developed by Nesje *et al.* (2000) for the peregrine falcon (*Falco peregrinus*), and subsequently used for kestrels (Hille *et al.*, 2003). GenBank accession numbers of the six microsatellite sequences are reported in Table 1. The polymerase chain reactions (PCR) were performed in a Perkin-Elmer, Gene Amp 2400 thermal cycler. The cycling conditions and number of cycles for all the primers are the same as those reported by Hille *et al.* (2003). All the reactions took place in a volume of 10 μl, containing 0.5 μl of DNA solution and a mix with 0.3U of Taq polymerase (GoTaq DNA Polymerase, Promega, Madison, WI), 1.8 $\mathrm{mmol \cdot l^{-1}}$ $MgCl_2$, 0.15 $\mathrm{mmol \cdot l^{-1}}$ dNTP, 2 $\mathrm{pmol \cdot l^{-1}}$ of each primer, and 0.5 $\mathrm{pmol \cdot l^{-1}}$ of the upper primer labelled with Cy5 dye. Allele sizes were determined with capillary denaturant electrophoresis, using a Bechman & Coulter CEQ 2000 automatic sequencer and a CEQ DNA size standard 400 as molecular weight markers.

Statistical analyses

The difference in genetic variability between the two groups was assessed by comparing the observed heterozygosity for each locus using the Wilcoxon's matched-pair test (Z). Statistical analyses were performed with the software SPSS 10.0 for Windows (SPSS, 2000). Arlequin 2.0 (Schneider *et al.*, 2000) was used to calculate observed heterozygosity (H_o), expected heterozygosity (H_s), and deviations from Hardy-Weinberg equilibrium within the early and late groups. Genetic differences between the groups were assessed using F_{ST} calculated according to Weir and Cockerham (1984). Genetic differentiation was calculated following Fisher's method with GENEPOP (Raymond and Rousset, 2000). The F_{ST}-values were also standardized following Hedrick (2005). FSTAT 2.9.3 (Goudet, 2001) was used to evaluate the overall departure from Hardy-Weinberg equilibrium and linkage disequilibrium for each pair of loci when the two samples were pooled, as well as the confidence limits of F_{ST}. GENEPOP was used to evaluate Rho_{ST}. STRUCTURE 2.1 (Pritchard *et al.*, 2000) was used to determine the probabilities of individuals belonging to the early versus late breeding groups.

RESULTS

Genotypes of the 40 (20 early and 20 late) nestlings were initially determined using all six loci. The NVH fp54 locus, however, showed low or non-amplifying alleles (null alleles), and so this locus was not considered further. Subsequent comparisons between early- and late-hatched chicks were therefore based on the remaining five loci (Table 1). The number of alleles per locus ranged from 3 to 12 and several alleles were unique to either early or late breeders, as observed for the loci NVH fp79-4 and NVH fp92-1. Both groups (early- and late-hatched chicks) had five polymorphic loci and a similar mean number of alleles per locus (early = 5.2; late = 5.4). No locus pairs showed significant linkage disequilibrium and the five loci were therefore considered to be statistically independent. Significant departures from Hardy-Weinberg equilibrium were not observed between groups ($P = 0.45$), but were evident among them (early, $P = 0.02$; late, $P = 0.05$). Observed heterozygosity did not differ between groups (mean value: early = 0.36 ± 0.09; late = 0.42 ± 0.12; $Z = 0.73$, $n = 5$, $P = 0.47$), suggesting no differences in genetic variability.

Table 1. Locus name, Gene Bank accession number, range in number of base pairs, number of alleles, and observed and expected heterozygosity for early- and late-hatched nestlings

	Early				Late				Over samples
Locus	Base range size	No. alleles	Observed H_o	Expected H_s	Base range size	No. alleles	Observed H_o	Expected H_s	H_s
NVH fp13	92–100	4	0.15	0.56	92–96	3	0.45	0.63	0.50
NVH fp31	132–142	6	0.55	0.80	134–142	5	0.60	0.79	0.80
NVH fp79-4	129–149	6	0.60	0.77	129–155	12	0.75	0.89	0.36
NVH fp89	117–123	4	0.25	0.31	117–123	4	0.25	0.67	0.72
NVH fp92-1	101–115	6	0.25	0.74	101–111	3	0.05	0.73	0.61
Mean		5.2 ± 0.49	0.36 ± 0.09	0.64 ± 0.09		5.4 ± 1.69	0.42 ± 0.12	0.74 ± 0.05	
Over loci									0.60

Early- and late-hatched chicks, and therefore their early- and late-breeding parents, were significantly different across the five microsatellite loci (Fig. 3). The mean F_{ST}-value was 0.058, while the standardized F_{ST}-value was 0.32. Rho_{ST} was 0.0038. Although the 95% confidence limits for F_{ST} overlapped zero (−0.07 and 0.130), the test for genetic differentiation was highly significant ($\chi^2 = 34.24$, d.f. = 10, $P = 0.0002$). A *post-hoc* assignment test in STRUCTURE based on the given population information correctly assigned all the individuals to their population of origin.

DISCUSSION

Genetic differences, whether for neutral loci such as microsatellites or for fitness-related traits, are expected as a consequence of a spatial separation between groups of individuals that show some dispersal limitation. This geographical contribution to population structure has long been accepted for large spatial scales, and has recently been shown for some species at quite small spatial scales (Blondel *et al.*, 1999; Partecke *et al.*, 2004; Garant *et al.*, 2005; Moore *et al.*, 2005). A recent example has been documented in the great tit (*Parus major*), in which genetic differences in body mass were caused by the non-random dispersal of fledglings as a consequence of small-scale density differences (Garant *et al.*, 2005). Moreover, small-scale genetic differentiation can be relatively rapid, bringing a population to diverge during a single colonization event, as observed in the blackbird, *Turdus merula* (Partecke *et al.*, 2004). Some of these spatial differences may be related in part to differences in reproductive timing between locations. For example, two populations of rufous-collared sparrows (*Zonotrichia capensis*) that were 25 km apart but showed different breeding times were found to differ at DNA microsatellites (Moore *et al.*, 2005). The two adjacent blue tit (*Parus caeruleus*) populations studied by Blondel *et al.* (1999) also showed different breeding times. A logical extension of these findings is that different breeding times might also allow for genetic differences to arise in sympatry, as documented in the present study.

Our results appear to show genetic differences in neutral markers between early- and late-breeding birds in sympatry. In our kestrel population, the observed differences were between broods raised in the same environment, often at very short distances from each other. These broods hatched from eggs laid at the beginning or end of the breeding period, and were separated in time by at least 1 month. The genetic differences are therefore most likely to be due to differences in breeding time. Since the microsatellite loci themselves are neutral with respect to selection, the differences are likely to have arisen due to (1) genetic drift between partially reproductively isolated breeding groups or (2) linkage between microsatellite loci and loci under differential selection between early and late breeders. The second scenario is supported by the fact that several of the loci showed substantial differences and several showed none (Table 2). Either scenario, however, is reflective of limited gene flow between early and late breeders.

The F_{ST}-value we found between early and late clutches is high compared with that in salmonid fishes, the only available data for vertebrates (Hendry and Day, 2005). Although it is not possible to compare our F_{ST}-value of 0.058 with those of other bird species, the value can be considered quite high, suggesting a strong differentiation between early and late breeders.

The genetic difference between early and late breeders is most likely the result of assortative mating-by-time. That is, individuals breeding at the extremes of the season produce offspring that breed at similar times. In this way, the progeny of early breeders mate with the progeny of other early breeders, whereas the progeny of late breeders mate with the

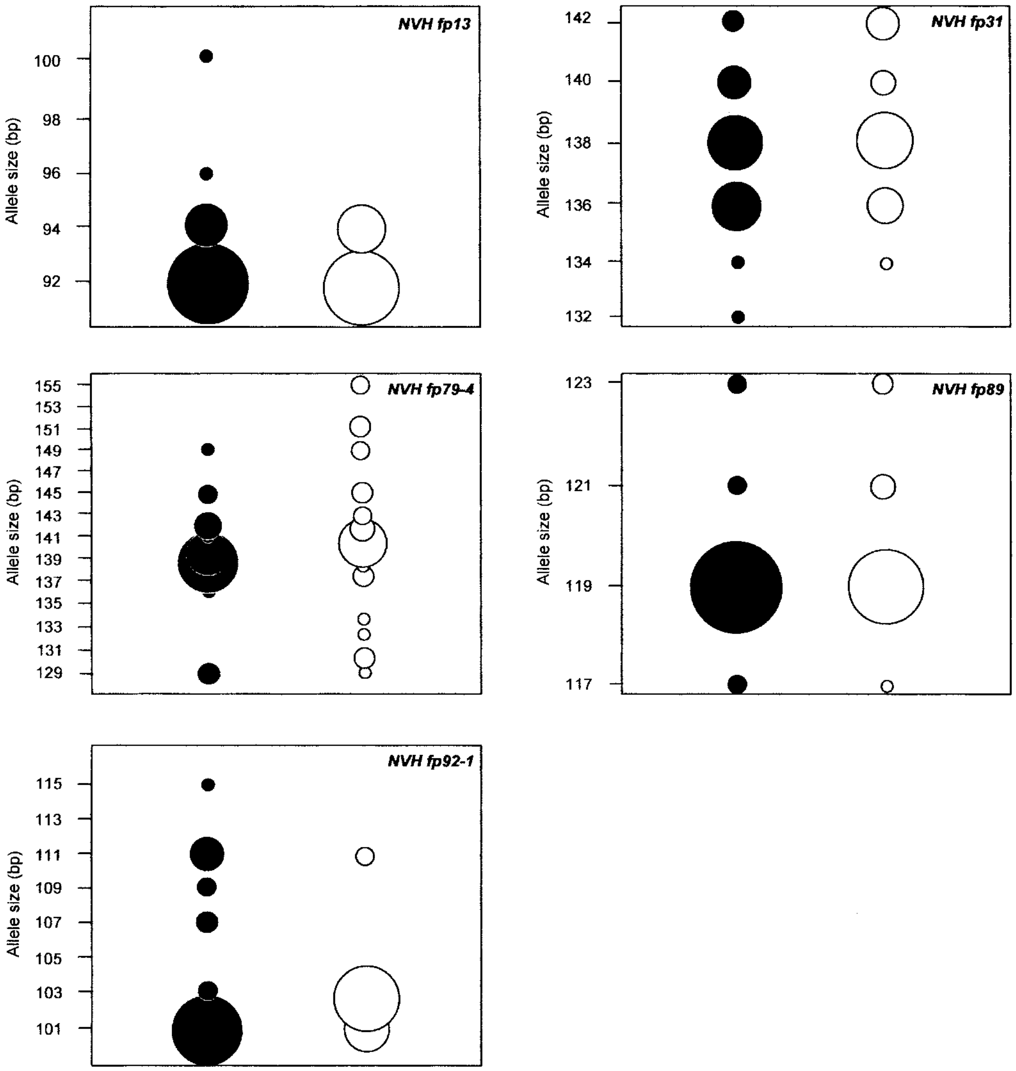

Fig. 3. Distribution of allele frequencies at the different loci in early (black) and late (white) nestlings.

Table 2. Locus-specific values for F_{ST}, Rho_{ST}, standardized F_{ST} (Hedrick, 2005), and genetic differentiation

Locus	F_{ST}	Standardized F_{ST}	Rho_{ST}	Genetic difference P-values
NVH fp13	−0.02	−0.09	0.0032	0.892
NVH fp31	0.06	0.24	0.1606	0.001
NVH fp79-4	0.12	0.48	0.0073	0.473
NVH fp89	−0.02	0.08	0.0017	0.683
NVH fp92-1	0.10	0.64	0.0149	0.0001

progeny of other late breeders. This then allows genetic differences to accrue with respect to breeding time. The possibility of assortative mating-by-time is further strengthened for kestrels by the very low incidence of extra-pair paternities (Korpimäki *et al.*, 1996). Assortative mating-by-time has also been documented for the blackcap *Sylvia atricapilla* (Bearhop *et al.*, 2005), but no tests have yet been made for genetic differences in that species. No other studies appear to have tested for genetic differences between early and late breeders in birds, but such differences are commonly observed in other species with heritable breeding times (for a review, see Hendry and Day, 2005).

Early studies on kestrels emphasized a presumed advantage to early breeders because they can produce larger and healthier broods than late breeders, thereby achieving greater reproductive success (Daan and Dijkstra, 1988). Moreover, it has been suggested that male kestrels hatched from early pairs have a higher probability of maturation and an earlier recruitment into the population than male kestrels hatched from late pairs (Daan *et al.*, 1990). In short, most studies of kestrels in particular, and birds in general, have disregarded the role of late breeders, and have not considered possible adaptive advantages of breeding late as an alternative strategy (Price *et al.*, 1988). We suggest that to breed early or late could represent two different strategies that might have evolved to maximize breeding success across a period with changing weather conditions and food availability. In the Mediterranean zone, the late period of the breeding season is meteorologically more constant than the early period, and sometimes late breeders can benefit from this situation (Costantini *et al.*, 2005). It appears that natural selection, while favouring pairs laying many eggs at the beginning of the season when food conditions are optimal, at the same time maintains late breeding pairs that lay fewer eggs. Although fewer eggs in late breeders thus may be adaptive, the specific reason underpinning this pattern has not yet been investigated.

In the present study, we compared birds breeding at two extremes of the breeding period, ignoring those with intermediate breeding times. We therefore cannot determine whether (1) a sharp genetic distinction occurs at some point during the breeding season, (2) more than two distinct groups are present, or (3) reproductive isolation is continuous, as in the idea of ‘isolation-by-time’ (Hendry and Day, 2005). These questions arose after we carried out the study and will be addressed in future work.

In conclusion, we documented the existence of genetic differentiation between chicks from early (larger) clutches and chicks from late (smaller) clutches. Our findings suggest it is possible that genetic differentiation may occur in a population of birds living sympatrically. Further genetic studies are needed to analyse the genotypes of the pairs breeding in other periods of the breeding season. Behavioural observations are also required to assess the importance of assortative mating-by-time as a possible mechanism for the appearance and maintenance of hereditability in breeding time. Furthermore, as the timing of breeding is under natural selection and the thermal conditions can greatly affect the timing of breeding of some species, it is possible that climate changes could modify the structure of bird populations (Crick *et al.*, 1997). Our results thus provide new elements to understand the breeding phenology of birds and the evolutionary dynamics of breeding time as a consequence to climate changes (Crick and Sparks, 1999).

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