THE ROYAL SOCIETY

Received 21 July 2003
Accepted 21 August 2003
Published online 14 May 2004

Heterogeneity in stable isotope profiles predicts coexistence of populations of barn swallows *Hirundo rustica* differing in morphology and reproductive performance

Anders Pape Møller[1*] and Keith A. Hobson[2]

[1]*Laboratoire de Parasitologie Evolutive, CNRS UMR 7103, Université Pierre et Marie Curie, Bâtiment A, 7ème étage, 7 quai St Bernard, Case 237, F-75252 Paris Cedex 05, France*
[2]*Canadian Wildlife Service, 115 Perimeter Road, Saskatoon, Saskatchewan, S7N 0X4, Canada*

Population studies assume that individuals belonging to a study population are homogeneous for natal and breeding origin, although this assumption is rarely tested. We tested for heterogeneity in stable-isotope profiles ($\delta^{15}N$, $\delta^{13}C$, δD) of feathers grown in the African winter quarters from a Danish breeding population of adult barn swallows, *Hirundo rustica*. Deuterium isotope values did not provide useful information on population segregation of wintering swallows in Africa. However, both $\delta^{15}N$ and $\delta^{13}C$ values showed a clearly bimodal distribution with 6% belonging to one category and the remaining birds belonging to another category, resulting in this population comprising three categories of birds. Adults belonging to the two categories of $\delta^{13}C$ isotope profiles differed weakly in morphology for several different characters. The frequency and the size of second broods differed between categories of $\delta^{13}C$ isotope profiles. Phenotypes of nestlings from the first brood in terms of tarsus length, body mass and T-cell response differed significantly between the two $\delta^{15}N$ isotope categories, suggesting that conditions during winter carried over to the breeding season at least as late as the first brood. Polymorphism can be maintained only if fitness is similar for birds from categories of isotope profiles. We suggest that fluctuating selection or migration-selection balance may maintain the observed polymorphism.

Keywords: connectivity; polymorphism; population subdivision; seasonal interaction; stable-isotope analyses

1. INTRODUCTION

Migratory animals live in different places at different times of the annual cycle, and attempts to understand their ecology and evolution rest upon an ability to link environmental conditions in these disparate parts of the range. Migratory connectivity describes the actual migration of individuals between breeding and non-breeding seasons, including stopover sites during the periods of migration (Webster *et al.* 2002). The degree of connectivity reflects the extent to which different reproducing populations form non-breeding populations during migration and in the winter quarters. The degree of population differentiation for connectivity has important implications for the ecology and evolution of migratory species.

Several different methods have been used to link the different parts of the annual cycle of different populations of a species. Direct methods to assign wintering origin of individuals to breeding populations include cases of morphologically distinct subspecies; direct information based on recoveries of individually ringed birds and satellite tracking of individuals (see the review in Webster *et al.* 2002). Recoveries may provide a biased estimate of winter distributions of different populations, because distribution and density of recoveries will depend on socio-economic status, human population density, literacy and many other factors. Indirect methods based on genetic and biogeochemical techniques may offer suitable solutions in the numerous cases where direct methods fail.

Previous studies using molecular markers have shown that such markers for different populations allow assignment of individuals (e.g. Burlando *et al.* 1996; Wennerberg 2001). Studies of the composition of a single population can be used to assign individuals to different populations based on maximum-likelihood approaches (Primmer *et al.* 2000; Hansen *et al.* 2001; Vázquez-Domínguez *et al.* 2001). Several studies of stable isotopic ratios have been able to identify movements of populations of animals because profiles reveal information on the wintering origin of individuals due to site-specific isotope profiles in tissue such as hair or feathers (see the review in Hobson 1999). This technique has been based largely on the use of stable-carbon and nitrogen isotope measurements to provide habitat-specific markers and more recently on the pattern of growing-season averages of deuterium in rainfall in North America (Hobson 2004). Thus, differences in locations where feathers or hair are grown will be reflected in differences in isotope profiles of those areas. Studies of such isotope profiles have been used to delineate separate parts of the breeding and wintering range of populations of birds (see Chamberlain *et al.* 1997, 2001; Hobson & Wassenaar 1997, 2001; Marra *et al.* 1998; Hobson *et al.* 2001; Meehan *et al.* 2001; Rubenstein *et al.* 2002). Some of these findings have corroborated direct evidence of separate breeding and wintering

*Author for correspondence (amoller@snv.jussieu.fr).

grounds for different subspecies of migratory birds (Chamberlain *et al.* 2001). Others have provided completely novel information about differentiation and levels of connectivity (e.g. Chamberlain *et al.* 1997; Hobson & Wassenaar 2001; Rubenstein *et al.* 2002). In other words, if breeding birds have different isotope values (and thus differ in wintering origins), then they may also have different natal origins. Thus, different methods can be useful tools when investigating the composition of breeding and wintering populations.

Sympatric individuals of a species are usually considered to belong to a single population. Thus, population studies in ecology and evolution habitually make this assumption, although this is an untested assumption. Even in species with migratory divides, or in species with neighbouring subspecies there is some gene flow across boundaries. Hence, any population will contain a certain number of immigrants, of which some have arrived from far away. Population assignment tests based on molecular data have been used to identify such immigrant individuals (see Primmer *et al.* 2000; Hansen *et al.* 2001; Vázquez-Domínguez *et al.* 2001). To the best of our knowledge, nobody has used stable isotopes to distinguish individuals of different wintering origin, when breeding in sympatry.

The aims of this study were to test if a breeding population of barn swallows, *Hirundo rustica*, was homogeneous for stable isotope profiles. We assessed whether stable isotope values had bimodal distributions. Second, because that was the case for two elements (C, N), the second aim of the study was to determine to what extent adults belonging to the two categories of birds differed in phenotype. Third, we tested whether conditions in the African winter quarters, as inferred from feather stable isotope values, had carry-over effects on reproduction by investigating the difference in reproductive success and offspring phenotype between isotope profile categories and so tested directly the Seasonal Interaction Hypothesis proposed by Webster *et al.* (2002). This hypothesis suggests that environmental conditions during migration and in winter may have carry-over effects to the breeding areas. We had no *a priori* predictions concerning the ability of each of these isotope profiles to explain variation in adult and nestling phenotype, respectively.

2. MATERIAL AND METHODS

(a) *Study species*

The barn swallow is a small (*ca.* 20 g), socially monogamous, semi-colonial passerine feeding on insects caught on the wing. Barn swallows feed on large insects, mainly Diptera, which are difficult to catch and hence require a considerable amount of manoeuvrability and agility (Møller 1994). Danish barn swallows arrive from the South African winter quarters in April–June, and return in August–October (Møller 1994). Males and females build a nest inside buildings, where the female lays a clutch of 1–7 eggs, usually 4–5 eggs, which are incubated for 14 days by the female. Both males and females feed the nestlings for 21 days and an additional 7 days after fledging. More than 60% lay a second clutch. Barn swallows breed solitarily or in colonies of up to more than 120 pairs. The outermost tail feathers are on average *ca.* 20% longer in adult males than in females in the Danish population, whereas other morphological characters are of similar size (Møller 1994). Long-tailed males enjoy a mating advantage, as demonstrated by observations and experiments (Møller 1988, 1994; Saino *et al.* 1997*a*).

(b) *Study area*

Data were collected at 12 different colonies in a study area of *ca.* 45 km^2 at Kraghede (57°12′ N, 10°00′ E), Denmark (see Møller 1994 for details). This study population of barn swallows has been the subject of a long-term study that began in 1970 (Møller 1994), although the present study is based only on data from 2000. The study site is an open farmland habitat with barn swallows breeding in barns and stables, and only rarely elsewhere under bridges and in culverts. The fields and scattered trees around farms comprise most of the area, with the exception of a few plantations, ponds, ditches and streams. The main crops are grass, barley, wheat and potatoes.

(c) *Collecting feathers*

Upon capture, we took a sample of feathers from the central part of the red throat badge of each adult individual. These feathers were originally collected to allow measurement of coloration in the laboratory under controlled conditions. There is no reason to believe that the isotope profile of these feathers would have been affected by coloration being partly determined by carotenoids. First, the concentration of carotenoids in feathers is very low (A. P. Møller, unpublished data). Second, a field study of preference for carotenoid-rich food during the breeding season by adult barn swallows revealed significantly fewer carotenoids in food compared with random insect samples collected from the same micro-sites at the same time as barn swallows were foraging (Ninni 2003). The feathers for each bird were stored at room temperature in a plastic bag in complete darkness until measurements were made. The feathers of the badge of adult barn swallows are moulted in the African winter quarters (Cramp 1988; A. P. Møller, unpublished data from South Africa, Namibia and Ghana).

(d) *Recording adult phenotype*

Adult barn swallows were captured in mist nets from the start of the breeding season. Barn swallows were captured regularly (at least weekly) from the start of arrival in spring. This allowed phenotypes to be measured at spring arrival from the African winter quarters before any effects of the breeding site on flexible phenotypic traits such as body mass, infestation with parasites and damage to tail feathers had appeared. If not a local recruit, they were provided with a numbered aluminium band and one or two colour bands used for individual identification. Phenotypic traits were measured in a standardized way by A.P.M. Beak length was measured to the start of feathering, beak height at the distal end of the nares and beak width at the commissure, all with a digital caliper to the nearest 0.01 mm. Tarsus length and keel length were measured with the same instrument. The length of the two outermost and the central tail feathers and the flattened wings was measured with a ruler to the nearest millimetre. Body mass was recorded to the nearest 0.1 g on a Pesola spring balance. Wing length was estimated as the mean of the right and the left wing, whereas tail length was estimated as the mean of the right and the left outermost tail feather. Wing asymmetry was estimated as the absolute difference between the length of the left and the right wing, whereas tail asymmetry was estimated as the absolute difference between the length of the left and the right outermost tail feather.

Wingspan was measured to the nearest millimetre on a ruler by stretching the wings to their maximum possible length. All

morphological characters were measured with high precision as shown by high repeatabilities (Møller 1991, 1994; Møller *et al.* 1995; unpublished data).

Individuals with broken or damaged feathers were excluded from the analyses. The tip of the outermost tail feathers of barn swallows is rounded, being composed of small barbs, and any broken barb leaves an irregular shape of the feather that is readily visible. The number of birds with damaged feathers was less than 3% in any given year, which is thus unlikely to have biased the analyses.

The number of fault bars in the tail and wing feathers was counted by observing the feathers against the sky and recording the number of transparent bars in the feathers (Møller 1989; Bortolotti *et al.* 2002).

(e) *Recording nestling phenotype*

When nestlings of the first brood were 12 days old, their tarsus length and body mass were recorded as described above.

At the age of 12 days, all nestlings were injected intradermally in the wing web (the patagium) with 0.2 mg of phytohaemagglutinin-P (PHA) (Sigma, L-8754, Sigma Inc., USA) in 0.04 ml isotonic saline (the antigen injection). The left wing web was injected with the same amount of saline only (a control injection). The thickness of wing webs was measured immediately before and 24 h after injection in inoculated sites using a pressure sensitive caliper known as a spessimeter (Alpa S.p.A., Milano, cod. SM112, Teclock, Japan) with an accuracy of 0.01 mm. The reaction to PHA was controlled for the effect of injection *per se* and thickening owing to saline injection. This was done by calculating the difference between the change in thickness of the right PHA-injected wing web (thickness 24 h after injection minus thickness before injection) and the corresponding change in thickness of the left wing web, only injection with saline. This procedure followed Saino *et al.* (1997*b*) in their study of barn swallows. The thickness of the wing web was measured three times before and after PHA injection, and the average of these three measurements was used in calculations. The repeatability of the wing web index was high and highly significant (Saino *et al.* 1997*b*).

(f) *Recording reproductive success and survival*

Nests were checked at least once per week, and daily around the presumed date of hatching and fledging. Seasonal production of offspring was simply the number of fledglings in the first and the second brood.

Approximately only two-thirds of all pairs raise two broods in a season, usually using the same nest or building a new nest next to the old one (Møller 1994).

Adult survival was recorded from recaptures or from re-sightings of colour-banded individuals in subsequent years. Capture–mark–recapture analyses of survival have shown that the capture probability of already-ringed birds is 98.5% (Møller & Szép 2002). Thus, simple records of birds provide a very good estimate of their true survival rate.

(g) *Isotope analyses*

Feathers were analysed blind for any data on individuals. Feathers were first cleaned of surface oils using several rinsings in a 2 : 1 chloroform : methanol solution followed by air drying in a fume hood for several days. Feather vanes were then subsampled and 0.1 mg weighed into small tin cups. These samples were then combusted in a Robo-Prep elemental analyser interfaced with a Europa 20 : 20 isotope-ratio mass spectrometer. The resultant CO_2 and N_2 gases were measured for their stable isotope ratios in δ-notation relative to PDB (Pee Dee Belemnite) and atmospheric AIR (nitrogen) standards, respectively, according to the formula presented in Hobson (1995). Based on thousands of measurements of an internal laboratory standard (egg albumen) we estimated measurement error to be 0.1‰ and 0.3‰ for $\delta^{13}C$ and $\delta^{15}N$ measurements, respectively.

Stable-hydrogen isotope analyses of feathers are complicated over conventional measurements of $\delta^{13}C$ and $\delta^{15}N$ because of the problem of uncontrolled isotopic exchange between samples and ambient water vapour (Wassenaar & Hobson 2000). Elsewhere, we describe how we routinely use keratin standards as a means of correcting for this effect so that the values reported here are equivalent to non-exchangeable feather hydrogen (Wassenaar & Hobson 2004). Briefly, this process involves the simultaneous measurement of unknowns with several replicates of three different keratin standards whose non-exchangeable δD values are known and which span the range of expected feather values. Algorithms generated from each run that relate δD values of unknowns to their expected non-exchangeable values are then used on a run-by-run basis.

Stable-hydrogen isotope measurements on feathers and keratin standards were performed on H_2 derived from high-temperature flash pyrolysis of feathers and CF-IRMS. Pure H_2 was used as the sample analysis gas and the isotopic reference gas. A Eurovector 3000™ high temperature elemental analyser (EA) with autosampler was used to automatically pyrolyse feather samples to a single pulse of H_2 gas (and N_2 and CO gas). The resolved H_2 sample pulse was then introduced to the isotope ratio mass spectrometer (Micromass Isoprime with electrostatic analyser) by an open split capillary. All δD results are expressed in the typical delta notation, in units of per mil (‰), and normalized on the VSMOW-SLAP (Vienna Mean Standard Ocean Water–Standard Light Antarctic Precipitation) standard scale. Repeated analyses of hydrogen isotope inter-comparison material IAEA-CH-7 (−100‰), routinely included as a check, yielded an external repeatability of better than ±1.5‰.

(h) *Statistical analyses*

Not all variables were measured for all individuals because some characters were broken or damaged. This resulted in slightly different sample sizes for the different tests.

Frequency distributions were used to investigate whether stable isotope profiles showed signs of bimodality. The isotope variables were subsequently dichotomized to allow them to be used as factors in analyses of variance. As there was no overlap in the frequency distributions between the two categories of individuals (figure 1), this dichotomization could be performed unambiguously. We used a stepwise forward procedure using full factorial models to find the simplest model that provided the best fit to the data. Dependent variables were phenotypic traits of adults and nestlings and reproductive variables, whereas sex and the dichotomized $\delta^{13}C$ and $\delta^{15}N$ variables were used as factors.

We used the sequential Bonferroni correction to assess the tablewide type I error rate by adjusting the significance level downwards for the number of tests made (Holm 1979; Wright 1992). Strict application of this method severely reduces the power of tests (Wright 1992), but such sacrificial loss of power can be avoided by choosing an experiment-wise error rate higher than the usually accepted 5%. We used 10% as suggested by Wright (1992) and Chandler (1995).

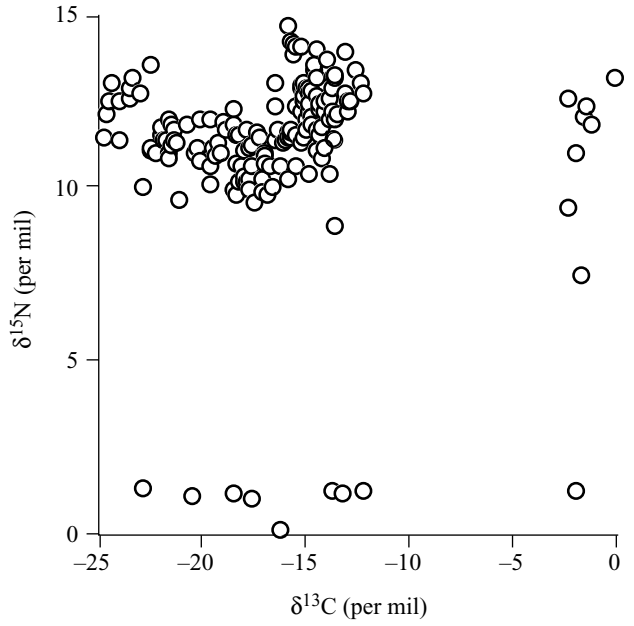

Figure 1. $\delta^{13}C$ for $\delta^{15}N$ in feathers from adult barn swallows.

3. RESULTS

(a) *Frequency distribution of and covariation among stable isotopes*

The feather stable-carbon and nitrogen isotope values showed bimodal frequency distributions. Both $\delta^{13}C$ and $\delta^{15}N$ values had a small fraction of individuals with extreme values, and individuals with small values for one isotope are not the same as individuals with small values for another isotope (figure 1).

The three isotopes were not significantly correlated ($\delta^{15}N$ and $\delta^{13}C$: $r = 0.003$, $n = 170$, $p = 0.957$; $\delta^{15}N$ and δD: $r = 0.050$, $n = 85$, $p = 0.206$; $\delta^{13}C$ and δD: $r = 0.014$, $n = 85$, $p = 0.900$). This implies that signals based on one isotope are independent of signals based on the two others.

Because δD values provided little information on population subdivision, we did not estimate this isotope for more than half of the total sample. We do not use this variable in the remaining part of this paper.

(b) *Adult phenotype and isotope profile*

We investigated whether the phenotype of adults was significantly related to $\delta^{13}C$ and $\delta^{15}N$ isotope signals, using ANOVAs with the dichotomized $\delta^{13}C$ and $\delta^{15}N$ variables and sex as factors. The results are summarized in table 1. We made a principal component analysis (PCA) to obtain an estimate of overall body size. Using a varimax rotation we found an eigenvalue of 2.808 for PC1, explaining 23.4% of the variance. PC1 was strongly positively correlated with wing length ($r = 0.880$), wingspan ($r = 0.916$), tail length ($r = 0.730$) and keel length ($r = 0.614$), suggesting the assumption that this component reflects body size. We found weak significant differences for beak height, beak width, tail length and PC1 as an overall measure of body size for isotope profile (table 1). Most of these significant differences were recorded for $\delta^{13}C$ values, and only beak height was related to $\delta^{15}N$ values. After sequential Bonferroni correction only two effects for morphology remained significant: the interaction between $\delta^{13}C$ values and sex for bill width and tail length.

Fault bars appear in bird feathers as a consequence of stressful conditions during moult. The presence of fault bars was significantly related to $\delta^{15}N$ values, with more fault bars in individuals with small $\delta^{15}N$ values (i.e. among individuals belonging to the 'rare' $\delta^{15}N$ group) (table 1).

Barn swallows breed in colonies that differ in size. Colony size differed between individuals of the two groups of $\delta^{13}C$ profiles, with colony size being smaller in the 'rare' $\delta^{15}C$ and the 'rare' $\delta^{15}N$ group (table 1).

(c) *Reproduction and stable isotope profile*

Laying date for the first clutch did not vary significantly for isotope profile ($\delta^{13}C$: $F = 2.566$, d.f. = 1, $p = 0.112$; $\delta^{15}N$: $F = 0.215$, d.f. = 1, $p = 0.807$).

Seasonal reproductive success was not significantly related to isotope profiles ($\delta^{13}C$: $F = 3.633$, d.f. = 1, $p = 0.059$; $\delta^{15}N$: $F = 0.336$, d.f. = 1, $p = 0.715$). However, the presence of a second brood decreased with increasing $\delta^{13}C$ (logistic regression: $\chi^2 = 6.363$, d.f. = 1,146, $p = 0.012$, slope (s.e.m.) = -0.096 (0.040)). Likewise, the number of fledglings in the second brood differed significantly between the two categories of $\delta^{13}C$ profiles, with the 'rare' $\delta^{15}C$ group having more fledglings ($F = 3.576$, d.f. = 1,62, $p = 0.034$).

(d) *Nestling phenotype and stable isotope profile*

Tarsus length differed significantly between the two categories of $\delta^{15}N$ profiles, with nestlings in the 'rare' group having longer tarsi than nestlings from the 'common' group (figure 2*a*). Mean tarsus length differed by on average 0.9%. Nestling body mass showed a difference for $\delta^{15}N$ (figure 2*b*). Nestlings in the 'rare' group were heavier than nestlings in the 'common' group, differing, on average, by 13.4%. However, the strongest response was found for T-cell-mediated immune response (figure 2*c*). Nestlings from the 'rare' group had a weaker T-cell response than nestlings from the 'common' group, differing on average by 26.7%.

Because the three phenotypic characters of nestlings are positively correlated, we investigated the independent relationships between nestling phenotype and $\delta^{15}N$ category, while entering the other two characters as covariates. The analyses confirmed that there were significant and independent relationships between nestling phenotype and $\delta^{15}N$ category even when controlling for the effects of the other two variables (table 2).

4. DISCUSSION

The main findings of this study were as follows: (i) stable-carbon and nitrogen isotope profiles of barn swallows from a Danish population showed bimodal distributions with a small fraction of adults differing from the rest; (ii) adult barn swallows belonging to these isotope categories differed in terms of morphology, the frequency of fault bars, and colony size where breeding; (iii) adult barn swallows from the $\delta^{13}C$ isotope categories differed in terms of frequency and size of second broods; and (iv) the phenotype of nestlings from first broods differed significantly between the $\delta^{15}N$ isotope categories of parents. We had no *a priori* predictions concerning the ability of each

Table 1. Stepwise forward models of adult barn swallow phenotype in relation to $\delta^{13}C$ category, $\delta^{15}N$ category, sex and their interactions as predictors.

factor	parameter	d.f.	SS	*F*	*p*
bill length	AIC = 347.269	d.f. = 167	$r_2 = 0.016$		
$\delta^{13}C$	−15.192	1	7866.227	2.746	0.099
bill height	AIC = 984.227	d.f. = 167	$r_2 = 0.030$		
$\delta^{15}N$	−6.726	1	1702.380	5.092	0.025
bill width	AIC = 328.827	d.f. = 163	$r_2 = 0.086$		
$\delta^{15}N$	−8.171	2	10857.890	2.163	0.118
$\delta^{13}C$	−33.621	2	18377.29	3.661	0.028
sex	−52.962	2	33056.280	4.390	0.0053[a]
$\delta^{15}N \times$ sex	−13.899	1	6827.353	2.720	0.101
$\delta^{13}C \times$ sex	34.607	1	16534.160	6.587	0.011[a]
tail length	AIC = 651.442	d.f. = 163	$r_2 = 0.646$		
$\delta^{13}C$	3.487	2	325.671	3.372	0.037
sex	−13.858	2	13987.17	144.830	0.0001[a]
$\delta^{13}C \times$ sex	−4.830	1	324.384	6.718	0.010[a]
body mass	AIC = 909.790	d.f. = 166	$r_2 = 0.143$		
$\delta^{15}N$	−3.156	2	1250.027	2.938	0.056
sex	−1.536	2	5290.476	12.435	0.0001[a]
$\delta^{15}N \times$ sex	4.244	1	651.720	3.064	0.082
PC 1	84.892	d.f. = 63	$r_2 = 0.412$		
$\delta^{13}C$	0.338	2	2.663	2.266	0.107
sex	−1.045	2	65.610	55.845	0.0001[a]
$\delta^{13}C \times$ sex	−0.438	1	2.662	4.531	0.025
fault bars	AIC = 340.683	d.f. = 167	$r_2 = 0.038$		
$\delta^{15}N$	−0.153	1	0.879	6.677	0.011[a]
colony size	AIC = 728.157	d.f. = 166	r_2=0.038		
$\delta^{15}N$	−1.764	1	113.991	1.561	0.213
$\delta^{13}C$	−3.583	1	425.883	5.831	0.012[a]

[a] Statistically significant after sequential Bonferroni correction for eight tests.

of these isotope profiles to explain variation in adult and nestling phenotype, respectively. We will discuss each of these findings in the following paragraphs.

Contrary to the strong latitudinal relationships observed between growing-season precipitation δD and the δD values of feathers grown in North America, we found there to be relatively high variability in δD values of swallow feathers grown on the wintering grounds in Africa. We also found no relationship between feather δD values and their $\delta^{13}C$ and $\delta^{15}N$ values, both indicators of local environmental conditions and food webs. The mean feather δD value of −30.3‰ (±19.4‰) corresponds reasonably well with feather isotopic distributions expected from South Africa for the months of August through to November (i.e. *ca.* −5‰ after accounting for a feather to precipitation fractionation factor of −25‰; Wassenaar & Hobson 2001) based on our analysis of regional maps of stable isotope data from the Global Network for Isotopes in Precipitation (GNIP) over the 1961–1999 period (IAEA 2001). We suspect that the high δD variance in our sample represents high local variability in feather δD values possibly associated with local dynamics of deuterium in water bodies over the wintering ranges of our swallow population (e.g. Zeigler 1988). For the purposes of this paper, we consider our inferences based only on the better-understood $\delta^{13}C$ and $\delta^{15}N$ associations between bird tissues and habitat conditions in Africa to be the most parsimonious.

We propose that the rare and common isotope categories of barn swallows winter in different parts of Africa. This assumption is based on the observation that isotope profiles differ between different parts of Africa (Cramp 1988), and that barn swallows from different breeding areas winter in different parts of Africa. Hence, barn swallows breeding in some parts of Europe are likely to consist of birds with a mix of winter ranges. Barn swallows from the Western Palaearctic winter mainly in sub-Saharan Africa (Cramp 1988). Populations from different parts of Europe use different winter quarters with a match of the west to east gradient in breeding distribution and wintering distribution, and northern breeding populations wintering further south than southern breeding populations (A. P. Møller, unpublished data). The present study provided evidence of a breeding population with clearly dichotomous isotope profiles. A small fraction of the breeding population differed markedly from most of the population, and extreme individuals for one isotope were not generally extreme individuals for the other isotope (figure 1). The distributions of both $\delta^{13}C$ and $\delta^{15}N$ values had very large variances, with a 12.5‰ for $\delta^{13}C$ and 7.5‰ for $\delta^{15}N$. In comparison, variation in $\delta^{13}C$ values for the entire breeding range of eastern North America is only 5.5‰ (Rubenstein *et al.* 2002), whereas it is 6‰ for $\delta^{15}N$ (D. R. Rubenstein, personal communication). This suggests that: (i) there is a huge variation in $\delta^{13}C$ and $\delta^{15}N$ values across Africa; or that (ii) not all birds winter in the

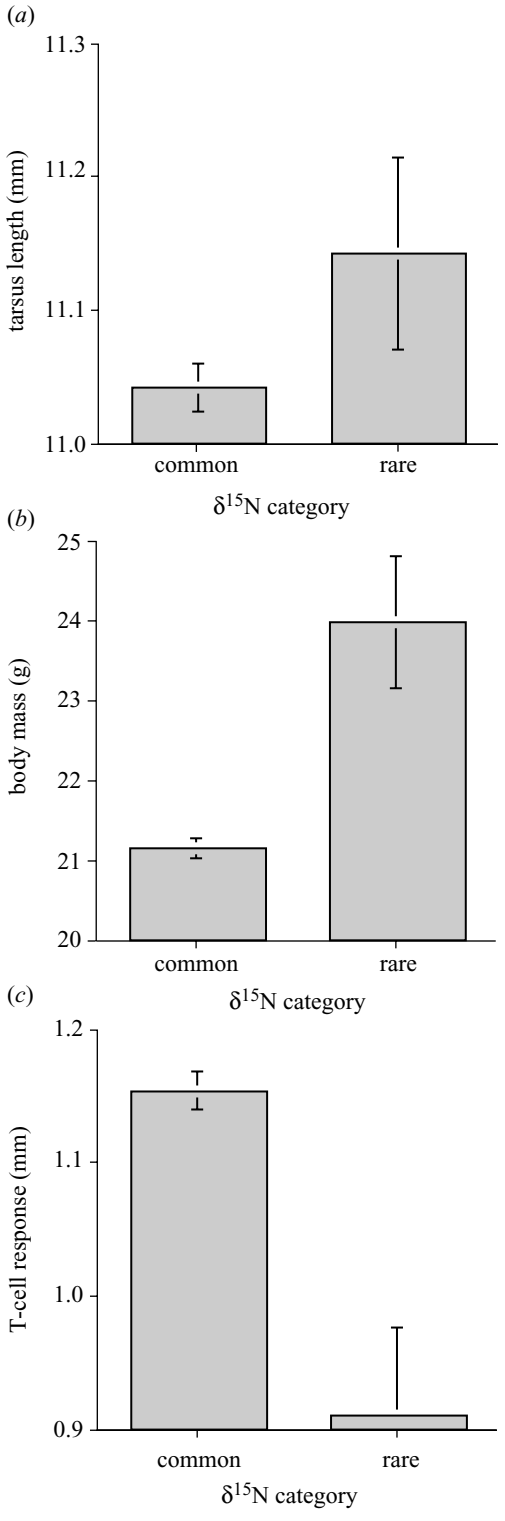

Figure 2. Phenotype of nestling barn swallows in relation to the $\delta^{15}N$ profile of their parents. 'Common' had more than 6.0 $\delta^{15}N$, whereas 'rare' had less than 1.5 $\delta^{15}N$. (*a*) Tarsus length. (*b*) Body mass. (*c*) T-cell response. Values are means (s.e.m.). Sample sizes are 130 for 'common' and 10 for 'rare'.

same part of Africa. Because the barn swallow moults in the African winter quarters (Møller 1994), the simplest interpretation of these findings is that at least two sympatric populations of barn swallows with different winter quarters co-occur in the Danish breeding population. Such cryptic sympatry of breeding populations revealed by isotope profiles alone has never been reported previously for migratory birds. The exact location of the different winter quarters remains to be determined, although winter recoveries of Danish barn swallows have been mainly in South Africa. Because the percentage of aberrant phenotypes amounted to only 6% of the adults, the total number of these individuals was relatively small. Hence, the results of the statistical tests should be considered with caution.

If the two groups of individuals differing with respect to stable isotopes were differentiated, we should be able to identify phenotypic differences. Analyses of phenotypic differences between isotope profile groups provided weak evidence of such differentiation (table 1). Once the statistical tests had been corrected for multiple tests using Bonferroni correction, only two weak interactions between $\delta^{13}C$ and sex remained statistically significant.

If the winter quarters differ in suitability, we should expect condition to differ among individuals belonging to the isotope categories. Indeed, when we investigated the frequency of fault bars in feathers in the categories of $\delta^{15}N$, we found a significant difference (table 1). Because fault bars appear as a consequence of stressful conditions (Møller 1989; Bortolotti *et al.* 2002), we can infer that the categories of barn swallows may have experienced different wintering conditions in the two areas. This result is contrary to previous stable isotope studies that have found relatively higher $\delta^{15}N$ values to be related to nutritional stress in birds (Hobson & Clark 1992; Hobson *et al.* 1993). Similarly, several researchers have associated relatively larger $\delta^{15}N$ values in tissues of drought-tolerant herbivores relative to water-dependent species in east Africa (Ambrose & DeNiro 1986, 1987; Sealy *et al.* 1987), or they have observed general foodweb relationships between $\delta^{15}N$ values and xeric environmental conditions in Africa (Heaton 1987; Koch 1998).

Environmental conditions in the winter quarters may have long-lasting effects on migratory birds that can carry over to the breeding season. If that is the case, then we should be able to find differences in phenotype between the two categories of adults differing in isotope profile. Indeed, breeding colony size differed significantly between these two categories (table 1). Likewise, the frequency of second broods and reproductive success in the second brood was related to $\delta^{13}C$ values. This is consistent with carry-over effects from wintering to the breeding season and so supports the Seasonal Interaction Hypothesis. Alternatively, the two categories of barn swallows (the rare and the common isotope phenotype) might represent individuals with different foraging strategies, even wintering in sympatry. We consider this possibility to be unlikely for several reasons. First, polymorphic foraging specializations are generally associated with polymorphic morphologies. That is the case in sex-specific foraging specializations, but also in other polymorphisms (Ligon 1968; Selander 1966; Shine 1989; Smith 1993). Thus, it seems difficult to reconcile a hypothetical case of foraging specialization with an absence of phenotypic specialization. Second, although differences in adult phenotype were related to differences in $\delta^{13}C$ values (table 1), differences in nestling phenotype were related to differences in $\delta^{13}N$ (figure 2). Thus, we would have to invoke two different kinds of foraging specialization to account for these differences.

Table 2. Analyses of covariance with nestling phenotype as the dependent variable, $\delta^{15}N$ category as a factor, and the two other nestling characters as covariates.

variable	d.f.	SS	F	p	parameter
tarsus length					
model:	$F = 3.183$	d.f. = 3,136	$p = 0.026$		
$\delta^{15}N$	1	2027.545	4.898	0.029	−8.492
body mass	1	2968.187	7.171	0.0083	−0.302
T-cell response	1	180.906	0.437	0.510	−0.072
body mass					
model:	$F = 16.341$	d.f. = 3,136	$p < 0.0001$		
$\delta^{15}N$	1	5339.220	23.440	< 0.0001	−12.955
tarsus length	1	1633.373	7.171	0.0083	−0.166
T-cell response	1	1009.004	4.430	0.0372	−1.168
T-cell response					
model:	$F = 14.679$	d.f. = 3,136	$p < 0.0001$		
$\delta^{15}N$	1	2494.480	9.776	0.0022	0.260
tarsus length	1	111.515	0.437	0.510	−0.044
body mass	1	1130.264	4.430	0.037	−0.188

The relationship between stable isotope profile and offspring phenotype was very strong. Tarsus length and in particular body mass and T-cell-mediated immune response differed between the two categories of parental $\delta^{15}N$ (figure 2). These effects of offspring phenotype were independent of each other, because ANCOVAs revealed significant relationships between nestling phenotype and isotope profile even when controlling for the two other phenotypic characters (table 2). Because nestling body mass is a reliable predictor of recruitment (Møller 1994), and since T-cell-mediated immune response predicts nestling survival (Merino *et al.* 2000), we can infer that the carry-over effects from winter quarters to the breeding season will have a long-lasting effect even on nestlings in the next generation. In addition, previous studies of T-cell-mediated immune response have shown that adults trade offspring quality against their own survival (Saino *et al.* 1999, 2002). The findings reported here imply that the trade-off between offspring immune response and adult survival may differ among population, perhaps because of differences in the impact of parasite-mediated natural selection.

Stable polymorphism requires that fitness benefits are equal, for example owing to fluctuations in the fitness benefits to the two categories of barn swallows in different years. Alternatively, migration–selection balance, with net immigration from another population being balanced by a selective disadvantage in the Danish breeding population, may account for the polymorphic winter distribution of the Danish barn swallows. Because the more rare population produced heavy offspring, but offspring with weak T-cell-mediated immune responses, it remains unclear whether the rare population is favoured or disfavoured selectively. The more rare fraction of the population for $\delta^{15}N$ tended to be found in smaller colonies. A possible interpretation is that the natal origin of the rare population for $\delta^{15}N$ is in an area with weak parasite pressure, with offspring allocating resources to body mass. Because the Scandinavian breeding populations have low population density (Cramp 1988), this may result in a relatively weaker parasite pressure. Therefore, a preliminary working hypothesis is that the rare Danish population originates from Scandinavia. This interpretation is supported by a recruit in the study population in 2001 being ringed as a nestling in Sweden.

K.A.H. was supported by grants from The Canadian Wildlife Service, Prairie and Northern Region. Patricia Healy assisted with the preparation of stable isotope samples. Analysis of carbon and nitrogen isotope samples was conducted at the Department of Soil Science, University of Saskatchewan, and deuterium samples at the National Water Research Institute, Saskatoon. The authors thank Len Wassenaar for assistance with deuterium isotope measurements of feathers.

REFERENCES

Ambrose, S. A. & DeNiro, M. J. 1986 The isotopic ecology of east African mammals. *Oecologia* **69**, 395–406.

Ambrose, S. A. & DeNiro, M. J. 1987 Bone nitrogen isotope composition and climate. *Nature* **325**, 201.

Bortolotti, G. R., Dawson, R. D. & Murza, G. L. 2002 Stress during feather development predicts fitness potential. *J. Anim. Ecol.* **71**, 333–342.

Burlando, B., Arillo, A., Spano, S. & Machetti, M. 1996 A study of the genetic variability in populations of the European woodcock (*Scolopax rusticola*) by random amplification of polymorphic DNA. *Ital. J. Zool.* **63**, 31–36.

Chamberlain, C. P., Blum, J. D., Holmes, R. T., Feng, X. H., Sherry, T. W. & Graves, G. R. 1997 The use of isotope tracers for identifying populations of migratory birds. *Oecologia* **109**, 132–141.

Chamberlain, C. P., Bensch, S., Feng, X., Åkesson, S. & Andersson, T. 2001 Stable isotopes examined across a migratory divide in Scandinavian willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) reflect their African winter quarters. *Proc. R. Soc. Lond.* B **267**, 43–48. (DOI 10.1098/rspb.2000.0964.)

Chandler, C. R. 1995 Practical considerations in the use of simultaneous inference for multiple tests. *Anim. Behav.* **49**, 524–527.

Cramp, S. (ed.) 1988 In *The birds of the Western Palearctic*, vol. V. Oxford University Press.

Hansen, M. M., Ruzzante, D. E., Nielsen, E. E. & Mensberg, K. L. D. 2001 Brown trout (*Salmo trutta*) stocking impact assessment using microsatellite DNA markers. *Ecol. Applic.* **11**, 148–160.

Heaton, T. H. E. 1987 The $^{15}N/^{14}N$ ratios of plants in South Africa and Namibia: relationship to climate and coastal/saline environments. *Oecologia* **74**, 236–246.

Hobson, K. A. 1995 Reconstructing avian diets using stable-carbon and nitrogen isotope analysis of egg components—patterns of isotopic fractionation and turnover. *Condor* **97**, 752–762.

Hobson, K. A. 1999 Tracing origins and migration of wildlife using stable isotopes: a review. *Oecologia* **120**, 314–326.

Hobson, K. A. 2004 Flying fingerprints: making connections with stable isotopes and trace elements. In *Birds of two worlds* (ed. P. Marra & R. Greenberg). Washington, DC: Smithsonian Institution Press. (In the press.)

Hobson, K. A. & Clark, R. W. 1992 Assessing avian diets using stable isotopes. II: factors influencing diet–tissue fractionation. *Condor* **94**, 189–197.

Hobson, K. A. & Wassenaar, L. I. 1997 Linking breeding and wintering grounds of neotropical migrant songbirds using stable hydrogen isotopic analysis of feathers. *Oecologia* **109**, 142–148.

Hobson, K. A. & Wassenaar, L. I. 2001 Isotopic delineation of migratory wildlife populations in North America: loggerhead shrikes. *Ecol. Applic.* **11**, 1545–1553.

Hobson, K. A., Alisauskas, R. T. & Clark, R. G. 1993 Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analyses of diet. *Condor* **95**, 388–394.

Hobson, K. A., McFarland, K. P., Wassenaar, L. I., Rimmer, C. C. & Goetz, J. E. 2001 Linking breeding and wintering grounds of Bicknell's thrush using stable isotope analysis of feathers. *Auk* **118**, 16–23.

Holm, S. 1979 A simple sequentially rejective multiple test procedure. *Scand. J. Statist.* **6**, 65–70.

IAEA 2001 *Isotope hydrology information system*. The ISOHIS database. Accessible at http://isohis.iaea.org.

Koch, P. L. 1998 Isotopic tracking of change in diet and habitat use in African elephants. *Science* **267**, 1340–1343.

Ligon, J. D. 1968 Sexual differences in foraging behavior in two species of *Dendrocopus* woodpeckers. *Auk* **85**, 203–215.

Marra, P. P., Hobson, K. A. & Holmes, R. T. 1998 Linking winter and summer events in a migratory bird by using stable-carbon isotopes. *Science* **282**, 1884–1886.

Meehan, T. D., Lott, L. A., Sharp, Z. D., Smith, R. B., Rosenfield, R. N., Stewart, A. L. & Murphy, R. K. 2001 Using hydrogen isotope geochemistry to estimate the natal latitudes of immature Cooper's hawks migrating through the Florida keys. *Condor* **103**, 11–20.

Merino, S., Møller, A. P. & de Lope, F. 2000 Seasonal changes in immunocompetence and mass gain in nestling barn swallows: a parasite-mediated effect? *Oikos* **90**, 327–332.

Møller, A. P. 1988 Female choice selects for male sexual tail ornaments in the monogamous swallow. *Nature* **322**, 640–642.

Møller, A. P. 1989 Viability costs of male tail ornaments in a swallow. *Nature* **339**, 132–135.

Møller, A. P. 1991 Sexual selection in the monogamous barn swallow (*Hirundo rustica*). I. Determinants of tail ornament size. *Evolution* **45**, 1823–1836.

Møller, A. P. 1994 *Sexual selection and the barn swallow*. Oxford University Press.

Møller, A. P. & Szép, T. 2002 Survival rate of adult barn swallows *Hirundo rustica* in relation to sexual selection and reproduction. *Ecology* **83**, 2220–2228.

Møller, A. P., de Lope, F. & Saino, N. 1995 Sexual selection in the barn swallow *Hirundo rustica*. VI. Aerodynamic adaptations. *J. Evol. Biol.* **8**, 671–687.

Ninni, P. 2003 The function of carotenoids in the barn swallow *Hirundo rustica*. PhD thesis, Université Pierre et Marie Curie, Paris, France.

Primmer, C. R., Koskinen, M. T. & Piironen, J. 2000 The one that did not get away: individual assignment using microsatellite data detects a case of fishing competition fraud. *Proc. R. Soc. Lond.* B **267**, 1699–1704. (DOI 10.1098/rspb.2000.1197.)

Rubenstein, D. R., Chamberlain, C. P., Holmes, R. T., Ayres, M. P., Waldbauer, J. R., Graves, G. R. & Tuross, N. C. 2002 Linking breeding and wintering ranges of a migratory songbird using stable isotopes. *Science* **295**, 1062–1065.

Saino, N., Primmer, C. R., Ellegren, H. & Møller, A. P. 1997*a* An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*). *Evolution* **51**, 562–570.

Saino, N., Calza, S. & Møller, A. P. 1997*b* Immunocompetence of nestling barn swallows in relation to brood size and parental effort. *J. Anim. Ecol.* **66**, 827–836.

Saino, N., Calza, S., Ninni, P. & Møller, A. P. 1999 Barn swallows trade survival against offspring condition and immunocompetence. *J. Anim. Ecol.* **68**, 999–1009.

Sealy, J. C., Merwe, N. J., van der Lee Thorp, J. A. & Lanham, J. L. 1987 Nitrogen isotope ecology in southern Africa: implications for environmental and dietary tracing. *Geochim. Cosmochim. Acta* **51**, 2707–2717.

Selander, R. K. 1966 Sexual dimorphism and differential niche utilization in birds. *Condor* **68**, 113–151.

Shine, R. 1989 Ecological causes for the evolution of sexual dimorphism: a review of the evidence. *Q. Rev. Biol.* **64**, 419–461.

Smith, T. B. 1993 Disruptive selection and the genetic basis of bill size polymorphism in the African finch *Pyrenestes*. *Nature* **363**, 618–620.

Vázquez-Domínguez, E., Paetkau, D., Tucker, N., Hinten, G. & Moritz, C. 2001 Resolution of natural groups using iterative assignment tests: an example from two species of Australian native rats (*Rattus*). *Mol. Ecol.* **10**, 2069–2078.

Wassenaar, L. I. & Hobson, K. A. 2000 Improved method for determining the stable-hydrogen isotopic composition (δD) of complex organic materials of environmental interest. *Environ. Sci. Technol.* **34**, 2354–2360.

Wassenaar, L. I. & Hobson, K. A. 2001 A stable-isotope approach to delineate geographical catchment areas of avian migration monitoring status in North America. *Environ. Sci. Technol.* **35**, 1845–1850.

Wassenaar, L. I. & Hobson, K. A. 2004 Rapid online technique for determinations of non-exchangeable δD of keratins for animal migration studies. *Isotopes Environ. Hlth Stud.* **39**. (In the press.)

Webster, M. S., Marra, P. P., Haig, S. M., Bensch, S. & Holmes, R. T. 2002 Links between worlds: unraveling migratory connectivity. *Trends Ecol. Evol.* **17**, 76–83.

Wennerberg, I. 2001 Breeding origin and migration pattern of dunlin (*Calidris alpina*) revealed by mitochondrial DNA analysis. *Mol. Ecol.* **10**, 1111–1120.

Wright, S. P. 1992 Adjusted P-values for simultaneous inference. *Biometrics* **48**, 1005–1013.

Zeigler, H. 1988 Hydrogen isotope fractionation in plant tissues. In *Stable isotopes in ecological research* (ed. P. W. Rundel, J. R. Ehleringer & K. A. Nagy), pp. 105–123. Berlin: Springer.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.