(A) Isoscape of δ^2H values (‰)

-160 -140 -120 -100 -80 -60 -40 -20 0

(B) Probability Surface for New Feathers ($\times 10^{-4}$)

0 0.018 0.064 0.14 0.22

(C) Probability Surface for Old Feathers ($\times 10^{-4}$)

0 0.018 0.064 0.14 0.22

Frontispiece. A comparison of stable hydrogen isotope values ($\delta 2H$) in sheath feathers and feathers from the previous molt cycle in the same sample of Bar-headed Geese. (A) is the isoscape of predicted δ^2H values for east-central Asia, with feather collection sites depicted as small black dots, small open circles and zones delineated by dashed lines correspond to important breeding sites/regions. The isoscape was used to generate a probability-of-origin surface for each feather in the sample, and (B) and (C) are the averaged probability surfaces for new and old feathers. The color pattern for (B) and (C) represents quartiles for the distribution of probabilities associated with new feathers.

WATERBIRDS

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Stable Isotopes Suggest Low Site Fidelity in Bar-headed Geese (*Anser indicus*) in Mongolia: Implications for Disease Transmission

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Abstract.—Population connectivity is an important consideration in studies of disease transmission and biological conservation, especially with regard to migratory species. Determining how and when different subpopulations intermingle during different phases of the annual cycle can help identify important geographical regions or features as targets for conservation efforts and can help inform our understanding of continental-scale disease transmission. In this study, stable isotopes of hydrogen and carbon in contour feathers were used to assess the degree of molt-site fidelity among Bar-headed Geese (*Anser indicus*) captured in north-central Mongolia. Samples were collected from actively molting Bar-headed Geese ($n = 61$), and some individual samples included both a newly grown feather (still in sheath) and an old, worn feather from the bird's previous molt ($n = 21$). Although there was no difference in mean hydrogen isotope ratios for the old and new feathers, the isotopic variance in old feathers was approximately three times higher than that of the new feathers, which suggests that these birds use different and geographically distant molting locations from year to year. To further test this conclusion, online data and modeling tools from the isoMAP website were used to generate probability landscapes for the origin of each

feather. Likely molting locations were much more widespread for old feathers than for new feathers, which supports the prospect of low molt-site fidelity. This finding indicates that population connectivity would be greater than expected based on data from a single annual cycle, and that disease spread can be rapid even in areas like Mongolia where Bar-headed Geese generally breed in small isolated groups. *Received 19 December 2014, accepted 26 March 2015.*

Describing and quantifying connectivity among subpopulations of migratory birds has emerged as an important theme in ornithology (Hobson *et al.* 2014; Stanley *et al.* 2014; Hallworth *et al.* 2015). Understanding how different regional breeding populations may intermingle during the migratory or wintering phases of their annual cycle can help prioritize conservation efforts and can inform continental-scale studies of disease transmission (Faaborg *et al.* 2010; Bridge *et al.* 2014; Runge *et al.* 2014). A case in point was the 2005 outbreak of the H5N1 strain of highly pathogenic avian influenza (HPAI) at Qinghai Lake in west-central China (Chen *et al.* 2005; Liu *et al.* 2005), which was followed by subsequent outbreaks in Russia, Western Europe, the Middle East, and Northern Africa. Since then, considerable research effort has been directed at understanding the role of wild birds in HPAI transmission, with particular attention to Bar-headed Geese (*Anser indicus*), which figured prominently in the Qinghai Lake outbreak. Commercial poultry farming and trade are often regarded as the means by which HPAI moves across the landscape (Sims *et al.* 2005; Roche *et al.* 2014), however; there is mounting evidence for the importance of wild birds as a long distance vector. For example, the recent infection records, which have established the presence of the H5N8 and H5N2 viral subtypes in North America (U.S. Department of Agriculture 2015a, 2015b; Verhagen *et al.* 2015)

Arguably, the best way of revealing connectivity among migratory populations is through the long-term and widespread use of satellite or cellular tracking technology. Although this technology is progressing rapidly, data from tracking studies are generally scarce due to the logistics of tagging operations and the cost of the equipment. Moreover, limited battery capacity, naturally

occurring mortality, and unexplained tag failure may also take a toll even after tags are deployed. For example, in two satellite telemetry studies of Bar-headed Geese, approximately half of the satellite tags deployed did not yield a data set for a full annual cycle (Takekawa *et al.* 2009; Hawkes *et al.* 2013). Faced with these and other limitations, there are few opportunities to look at year-to-year variation within individuals with regard to their breeding locations, molting sites, staging areas and breeding grounds.

Stable-isotope analysis provides an inexpensive means of obtaining low-resolution geographic information from individual birds (Hobson 2008), and with selective sampling of different tissues, isotope ratios can have a relevant time window ranging from a few days (e.g., stable isotopes in blood) to several months (e.g., stable isotopes in feathers). In this study, we use stable isotopes of hydrogen and carbon in feathers to evaluate the degree of molting-site fidelity demonstrated by Bar-headed Geese in central Mongolia. Our approach involved collection of both old and newly grown contour feathers from actively molting individuals to compare isotope ratios in two successive generations of plumage. We used the resulting data to test a null hypothesis of no difference in mean or variance in old and new feathers. This hypothesis is consistent with a scenario in which there is little or no yearly variation in environmental isotope ratios and the birds have high molting-site fidelity, giving rise to two feather generations with nearly identical isotopic compositions. Under these conditions, we would expect very limited mixing of different breeding populations and relatively limited potential for widespread disease transmission. Alternatively, if there was considerable mismatch between feathers from different plumage generations and if the discrepancy is not largely attrib-

utable to inter-annual variation in environ-mental isotope ratios, then we would infer that the birds move among different molting areas from year to year and that population connectivity may be higher than previously thought.

<div align="center">METHODS</div>

Study Area and Feather Collection

Except for a few large colonies, like the one at Quinghai Lake, Bar-headed Geese in China and India breed in colonies of a few hundred birds near high-altitude lakes (Carboneras 1992; Prins and van Wieren 2004). Breeding colonies in Mongolia rarely have more than 100 pairs, and colonies may nest on cliffs with no immediate access to large water bodies (Batbayar *et al.* 2014). Breeding occurs in early spring (May and June), after which the Bar-headed Geese may travel to a suit-able molting area, which is usually a large lake. Feather molt entails a rapid and complete replacement of the plumage, which renders the birds flightless for several weeks. During this time, their primary defense against predation is to take to the water. Therefore, Bar-Head-ed Geese must select molting habitats that provide both safety and sustenance. Presumably high-quality molting sites can host thousands of Bar-Headed Geese, even in Mongolia where breeding populations are sparse. When molt is complete the birds fly south, often going directly over the Himalayas to spend the winter in South Asia (Javed *et al.* 2000; Takekawa *et al.* 2009; Hawkes *et al.* 2011).

Contour feathers for this study were collected from 4 July to 30 July 2009 at nine lakes in central Mongolia (Table 1; Frontispiece). All birds were actively molting and flightless when captured. The feathers were col-lected opportunistically as part of a large scale band-ing effort. For this study, we accessed samples of five to 10 feathers from each of 62 adult birds (34 male and 28 female). Each sample of feathers contained either: 1) newly grown or growing feathers, distinguished by a keratinaceous sheath surrounding the base as well as a very clean outer edge along the vane; 2) old feathers, with noticeable wear along their edges and no sheath; or 3) a combination of new and old feathers. We as-sumed that all new feathers were grown in 2009 at the capture site and that the vast majority of old feathers were grown during the previous molt in 2008. Twenty-two of the samples had at least one old feather and one new feather, and for these samples we analyzed one feather from each age group. For the other 40 samples with entirely old or new feathers, we randomly selected a single feather for analysis. When choosing feathers for analysis, we gave preference to feathers for which aging was most certain.

Isotope Analyses

Isotope analysis focused on ratios of stable isotopes of hydrogen and carbon. Hydrogen isotope ratios vary geographically in a predictable manner due to patterns of deuterium retention in precipitation, which often allows for rough inferences about molting locations (Inger and Bearhop 2008). Carbon isotope ratios dif-fer according to the photosynthetic pathway (i.e., C3 or C4 photosynthesis) that serves as the primary source of organic carbon in an animal's diet (Hobson 1999). We expressed all isotope ratios in standard delta notation (δ^2H or δ^{13}C) where $\delta = [(\text{isotope ratio}_{\text{sample}}/\text{isotope ratio}_{\text{standard}}) - 1]$, with ratios shown as parts per thousand (‰) deviation from Vienna Standard Mean Ocean Wa-ter for hydrogen and Vienna Pee Dee Belemnite for carbon. Prior to analysis, all feathers were cleaned with dilute detergent and then a 2:1 chloroform:methanol solution following Paritte and Kelly (2009). For δ^2H analyses, we packed a 140-160 µg piece of feather vane into a 3.5 x 5 mm silver capsule. For carbon iso-tope analyses, we packaged a 350 µg piece of feather into a tin capsule. Isotope ratio measurements were performed at the University of Oklahoma with a Ther-moFinnigan Delta V isotope ratio mass spectrometer connected to an elemental analyzer (H analyses: TC/EA, Thermo-Finnigan; C analyses: CosTech elemental analyzer). To control for exchangeable hydrogen, hy-drogen isotope ratios were normalized according to Wasenaar and Hobson (2003), using the keratin stan-dards they established: chicken (*Gallus gallus*) feath-ers (-187‰), cow (*Bos taurus*) hooves (-138‰), and bowhead whale (*Balaena mysticetus*) baleen (-108‰). For additional details on our analysis methods, see Kel-ly *et al.* (2009) and Paritte and Kelly (2009). Carbon

Table 1. Molting sites (i.e., lakes) used as sampling locations.

Lake Name	Latitude	Longitude
Deed Ulaan Lake	49° 2' 58.73" N	101° 10' 49.99" E
Gun Lake	48° 24' 4.23" N	101° 53' 17.38" E
Khunt Lake	48° 12' 17.70" N	101° 41' 34.48" E
Khar-Us Lake	48° 24' 30.09" N	102° 14' 37.78" E
Duruu Lake	49° 1' 32.41" N	101° 13' 27.08" E
Khodoo Lake	48° 9' 12" N	99° 31' 36" E
Olon Lake	48° 4' 25.37" N	100° 21' 31.22" E
Terkhiin Tsagaan Lake	48° 8' 37" N	99° 36' 44" E
Khar Lake	48° 7' 51.56" N	99° 32' 12.82" E

analyses employed an in-house standard composed of Brown-headed Cowbird (*Molothrus ater*) feathers, which was calibrated with USGS40 (L-glutamic acid: $\delta^{15}N$ = -4.52‰, $\delta^{13}C$ = -26.39 ‰) and USGS41 (enriched L-glutamic acid: $\delta^{15}N$ = +47.57‰, $\delta^{13}C$ = +37.63‰) reference materials. Samples were measured in four different runs for each element, and in all cases precision was within ± 2‰ for hydrogen and 0.2‰ for carbon. We attempted to measure δ^2H or $\delta^{13}C$ for a total of 84 feathers, 44 of which were pairs of new and old feathers from the same individual, over the course of two auto-sampler runs for each element. Analyses failed for two δ^2H samples and one $\delta^{13}C$ sample, leaving us with 82 samples with δ^2H values, 83 samples with $\delta^{13}C$ values, and 21 individuals in which we measured both old and new feathers.

Statistical and Spatial Analyses

We used a simple one-way ANOVA to test for a difference in the mean isotope ratio between all old and new feathers. We also performed a similar but separate analysis in which we used a paired t-test to determine whether there was a difference in the mean isotope ratio between old and new feathers within birds for which we had feathers of both age classes. We also used Levene's test for unequal variance to determine whether variance within the two age classes of feathers was equivalent. All statistical analyses were performed in R statistical software (R Development Core Team 2014).

Investigation of how variation in isotope ratios translated into potential variation in geographical location was hampered by poor sampling of precipitation and lake water in central Asia. More specifically, we could not obtain sufficient precipitation isotope data to generate a model specific to the location and relevant time periods for linking feather isotope values to geographic locations. Hence, we employed a more general precipitation isoscape to generate probability surfaces associated with each feather sample so that we could compare distances between known feather collection sites and likely origins based on δ^2H. Using resources available on isoMAP (Bowen *et al.* 2014), we generated a long-term δ^2H isoscape for precipitation in an area (latitude range: 10° 48' N to 65° 0' N; longitude range: 67° 12' E to 138° 24' E) that surrounds our study site and includes all important breeding locations for Bar-headed Geese, based on distributions reported by Miyabayashi and Mundkur (1999) and Carboneras and Kirwan (2013), which are represented on the Frontispiece with dashed outlines and small circles, respectively. We used a geostatistical model (i.e., with kriging) that incorporated data from 69 stations collected from 1960 to 2009, and selected model parameters that comprised elevation, latitude squared, longitude squared, average temperature, and precipitation. Resolution was 0.5 degrees, and the isoscape and all relevant data and metadata are publically archived on isoMAP (job key 44905; see Bowen *et al.* 2014).

We generated geographical probability surfaces for each feather's origin as prescribed by Bowen *et al.* (2014), using R functions from Vander Zanden *et al.*

(2014). We used the δ^2H values from the new feathers along with predicted δ^2H values from the precipitation isoscape to generate a single discrimination value to account for changes in isotope ratios associated with the transfer from environment to animal tissues. Our sampling sites were clustered together, such that we could not achieve sufficient geographic representation to generate a transfer function based on regression of known-origin sample values against estimated precipitation values. Moreover, our δ^2H measurements from new feathers demonstrated a weak inverse relationship with predicted environmental isotope ratios (slope = -1.71, n = 28, R^2 = 0.20, P = 0.009; Fig. 1), which would render a nonsensical transfer function. The discrimination value we derived was 24.49‰ and had a standard deviation of 12.98. This value is consistent with the discrimination factor of 28‰ reported for Bar-headed Geese by Pérez *et al.* (2010). After applying the discrimination value to all feathers, we used the precipitation isoscape to assign probabilities of origin to all locations within the selected mapping extent while accounting for variation in environmental isotope values and among individual measurements (details in Vander Zanden *et al.* 2014).

To quantify how these probability surfaces related to potential variance in geographic locations, we compiled great-circle distances from each pixel in the isoscape to the collection location of each feather, and we calculated a weighted average for these distances using the probability for each pixel as the weighting parameter. Hence, a high concentration of likely locations (pixels) near the collection site would yield a low weighted average for distance, whereas a concentration of likely locations far
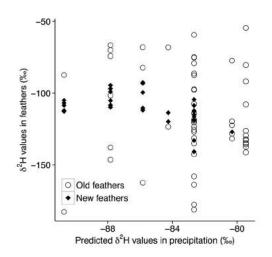

Figure 1. Predicted δ^2H values for collection sites based on the isoscape shown in the Frontispiece vs. raw δ^2H values for new and old feathers from Bar Headed-Geese in Mongolia. Note that although there were nine collecting sites, some of them were co-located on the same isoscape pixel and share the same predicted δ^2H value, resulting in only seven columns of points present.
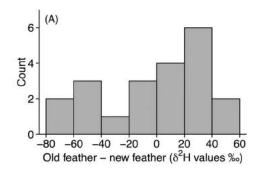
from the collection site would result in a high weighted average for distance. We used statistical analyses similar to those described above (ANOVA and paired t-test) to test for differences in the mean and variance for old and new feathers with respect to weighted distance values.

Results

There was no significant difference in δ^2H between old and new feathers from the same individuals (paired t-test: mean pairwise difference = -1.9‰, $t_{20} = 0.24$, $P = 0.82$). However, the distribution of the differences between old and new feathers varied considerably more than expected (Fig. 2). We note that 62% ($n = 21$) of the new/old feather pairings showing a difference in δ^2H large enough to exclude the old feather from the 95% credible interval for all new feathers (-135.5 to -93.0, $n = 28$). If the birds molted at the same location in 2008 and 2009, and if there was no inter-annual variation in environmental H isotope values (as the simi-larity of the yearly means suggests), then we would expect only 5% of the δ^2H values from 2008 to fall outside the 95% credible interval derived from the 2009 data. Analysis of all isotope data (not just paired data) yielded similar results. The variation in δ^2H values among all of the old feathers was about three times higher than that for all new feathers (old feathers: mean ± SD = -114.3 ± 32.3‰, $n = 54$; new feathers: -110.3 ± 11.2‰, $n = 28$; Fig. 3), and Levene's test for unequal variances indicated that this difference in variance was significant ($F_{1,80} = 21.7$, $P < 0.01$).

Weighted average distances were significantly smaller for new feathers relative to old feathers, and this difference held for comparisons of all feathers (new feathers: 1,628 ± 97 km; old feathers: 1,837 ± 329 km; ANOVA: $F_{1,80} = 10.9$, $P = 0.001$) and for pairwise comparisons (new feathers: 1,620 ± 96 km; old feathers: 1,931 ± 315 km; t-test: $t_{20} = 4.52$, $P < 0.001$). Variance was also greater among weighted average distances
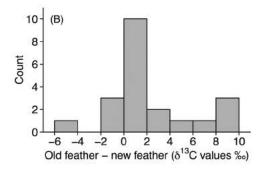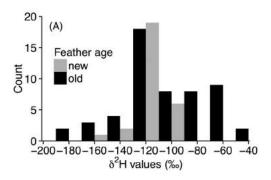

Figure 2. Distributions of pairwise differences in (A) δ^2H values and (B) δ^{13}C between old and new feathers from the same individual Bar-Headed Geese captured in Mongolia.
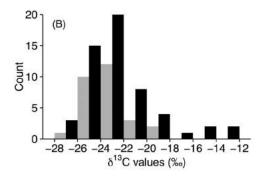

Figure 3. Distributions of (A) δ^2H values and (B) δ^{13}C values in new and old feathers from Bar-Headed Geese captured in Mongolia.

associated with old feathers as opposed to new feathers (Levene's test: $F_{1,80}$ = 18.5, $P <$ 0.001). Comparing the averaged probability surfaces for both old and new feathers reveals a much wider distribution of relatively high probability pixels for the old feathers (Frontispiece), which is consistent with the increased weighted average distances for old feathers. Although variation in environmental isotope ratios between years may account for some of the differences between old and new feathers, there was no clear directionality to the differences as would be expected if isotope values in precipitation and/or lake water differed from 2008 to 2009 (Fig. 1). The most likely explanation for the data is that a majority or near majority of these birds molted their old and new feathers in different locations.

The differences in $\delta^{13}C$ values between old and new feathers of the same bird demonstrated a small but significant positive shift, i.e., toward C4 photosynthesis (paired t-test: mean difference = 2.07‰, t_{20} = 2.58, P = 0.02). Almost all $\delta^{13}C$ values were less than -18‰. Assuming a general discrimination factor of 1.0 ± 1.03 for herbivorous waterfowl (Hahn et al. 2012), this range of values is reasonable for animals with a diet of carbon sources dominated by C3 photosynthesis, and it is possible that the difference between $\delta^{13}C$ in old and new feathers is largely due to inter-annual variation in environmental factors. However, four samples had $\delta^{13}C$ values that exceeded -18‰, which we interpret as a result of a diet dominated by C4 plants. The most likely source for C4 carbon within the species range would be agricultural crops, i.e., corn (*Zea mays*) and millet (*Pennisetum glaucum*). Considering all feathers, variances in $\delta^{13}C$ for old and new feathers were significantly different (Levene's test: $F_{1,81}$ = 6.0, P = 0.02), which is largely due to the feathers indicative of C4 carbon sources. With these feathers removed from the analysis, Levene's test was not significant at an alpha level of 0.05 ($F_{1,76}$ = 3.20, P = 0.08).

There was no correlation between the intra-individual differences for δ^2H and $\delta^{13}C$ (linear regression: n = 21, R^2 = 0.014, P = 0.60), but the four birds whose old feathers indicated C4 carbon sources did show large discrepancies between old and new feathers with respect to δ^2H. Absolute values of the δ^2H differences for these birds averaged 50‰, whereas the absolute differences for the other individuals averaged 26‰. Hence, birds that exploited agricultural food sources during the molting period that preceded the year of feather collection did not use the same molting grounds, and it is likely they molted in areas more distant from the sampling sites.

DISCUSSION

Our methods and the resulting isotope measurements are similar in many respects to the findings of Pérez et al. (2010), who examined isotope ratios in feathers collected from several waterfowl and shorebird species in Mongolia. However, our interpretation of the data differs considerably in light of our focus on migratory connectivity and disease dynamics. Pérez et al. (2010) sought latitudinal assignments based on δ^2H for unknown molting locations, and they concluded that broad-scale assignments are possible. Although they examined both old and new feathers as we did, Pérez et al. (2010) only considered the means for these two groups of samples and did not report variance for old and new feathers.

Our study focused on the question of whether Bar-headed Geese use the same molting locations year after year, and our data suggest that birds will readily alternate among distant molting locations in successive seasons. Hence, population connectivity for Bar-headed Geese may be higher than expected based on examination of a typical sampling of feather isotopes or a single year of tracking data. This finding has important implications for ongoing efforts to model disease dynamics (especially those of avian influenza) in relation to migratory birds. Isotope data cannot, of course, provide location data with accuracy equivalent to that of satellite tracking or band recoveries, but they can provide information on the geographic range represented within a con-

vergence of animals at a particular site. Bar-headed Geese breed both in small, isolated colonies consisting of a few dozen pairs as well as a few large groups such as the colony at Qinghai Lake, China, which comprises over 10,000 birds (Zhang and Hao 2009). The extent of their breeding range is poorly documented, but it is clear that their distribution is patchy, especially in the northern part of their range (Frontispiece). As such, the degree of connectivity among distant colonies would be presumed to be quite low as contact among individuals from different colonies would be minimal. However, our analyses reveal the need to calculate connectivity during the summer months based not only on breeding colonies, but also on molting congregations. Our data suggest that molting congregations provide an avenue for mixing of birds from distant breeding colonies, and that birds may move among different molting locations from year to year.

Although we attribute the isotopic differences in old and new feathers to use of different molting grounds in successive years, there are other possible explanations. Firstly, the birds sampled could not be aged (beyond determination of adult status). Hence it is possible that for some individuals, the old feathers sampled are natal plumage grown at breeding locations. However, this possibility does not entirely account for the wide discrepancy in variation of δ^2H in old and new feathers. It also is possible that some of the old feathers may have been replacements for feathers lost elsewhere in the annual cycle. Although this scenario is unlikely as an explanation for the overall patterns of variation observed, it may explain some of the extreme δ^2H values. For example, the highest observed δ^2H value (-54.5‰) is quite possibly from a feather grown on the wintering grounds in southern Asia. Year-to-year variation in lake water in our study region may also contribute to variation in feather isotope composition. If inter-annual variation in lake water was high and differed in direction among our study sites, then a high degree of variation in δ^2H values among the study sites might be explainable without assuming long-distance

shifts in molting locations. However, variation in δ^2H values appeared to be uniform across sites (Fig. 1), and the similarity in the mean δ^2H values for old and new feathers suggests little inter-annual variation between 2008 and 2009. Unfortunately, examining inter-annual variation in the study environment was problematic due to insufficient sampling. We found no data in the literature or in available databases that pertained directly to variation in δ^2H values in our study region. Studies of lakes in the Tibetan Plateau and in Eastern Siberia emphasize the importance of seasonal fluctuations in δ^2H, which can include enrichment on the order of 60‰ due to evaporation (Ichiyanagi *et al.* 2003; Tian *et al.* 2008; Pham *et al.* 2009). However, even under the assumption of extreme seasonal or inter-annual variation, it is difficult to conceive of a scenario that would generate variation in δ^2H values on the order of 120‰ for feathers molted by Bar-Headed Geese at the same lake (Fig. 1). Given that predicted δ^2H values varied by less than 10‰ among our study locations, it is also unlikely that merely shifting molting locations among the lakes in our study region could account for the observed variation in feather isotope ratios. Thus, we are led to conclude that Bar-headed Geese often undergo successive feather molts in geographically distant locations.

This conclusion agrees with field observations in Mongolia (Batbayar *et al.* 2014). Breeding colonies are often quite small (< 100 pairs) and are often spatially isolated. However, each molting site where our samples were collected hosts about 3,000 individuals every summer, which means that the birds at each molting area probably represent several breeding populations. Our isotope data suggest that the Mongolian molting grounds host birds that previously used molting areas within a geographic range that extends well beyond Mongolia.

The reasons for low molt site fidelity are unknown. It may be due to the condition of the habitat (ephemeral water bodies) or the degree of overcrowding at particular sites. Geese are flightless during much of their molting period, and they may need to search

broadly prior to molting for sufficient local resources meet the energetic and nutritional requirements of feather growth (Portugal *et al.* 2007). Bar-headed Geese may also alter their molt-migration strategy according to whether they breed successfully or not.

Despite considerable research, the role of wild birds in long-distance disease transmission is poorly understood, and it remains a topic of debate, especially with regard to avian influenza (Yasue *et al.* 2006; Altizer *et al.* 2011). Gilbert *et al.* (2006) noted a close spatial and temporal correspondence between the H5N1 outbreak locations and waterfowl migration routes across Central Asia to the Caspian Sea and Black Sea basins. Iverson *et al.* (2011) and Prosser *et al.* (2011) have also shown correlative evidence of HPAI spread across Central Asia via waterfowl migration. However, a significant number of studies have found no evidence of HPAI transmission via wild birds (Krauss *et al.* 2007; Takekawa *et al.* 2010; Samad *et al.* 2011), and some have argued for deemphasizing the role of wild-bird migration on disease HPAI transmission (Sims *et al.* 2005). We suggest that feather collection and isotope analyses provide a low-cost means of evaluating connectivity of migratory populations.

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