



Migratory dark-eyed juncos, *Junco hyemalis*, have better spatial memory and denser hippocampal neurons than nonmigratory conspecifics

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The evolution of migration in an animal population produces a suite of physiological, behavioural and cognitive adaptations. Migratory birds, in particular, require the ability to return annually to breeding and wintering sites after long journeys, and thus might be predicted to have evolved enhanced spatial memory. In a comparison of two sparrow subspecies that co-occur in winter, the migratory subspecies (dark-eyed junco, *Junco hyemalis hyemalis*) performed better than the nonmigratory subspecies (*J. h. carolinensis*) on a room-scale spatial memory test. The migratory juncos also had more densely packed hippocampal neurons than did nonmigrants. Among nonmigrants, we looked for hippocampal differences between birds that occupied two home ranges annually and those that remained on their breeding territory year-round, to determine whether migration, per se, is related to neuroanatomical differences. However, we were unable to reach any conclusions because of low statistical power. A denser hippocampus could be the basis for better spatial memory in migrant juncos. Further testing of spatial memory on a landscape scale is needed to strengthen this argument and to understand cognitive differences between migrants and nonmigrants.

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When populations become reproductively isolated, behaviours can undergo evolution just like other aspects of the phenotype that have some genetic basis. Cognitive abilities also would be expected to change as a result of natural selection. A well-studied example of this phenomenon is that of food-storing birds that must remember where items have been hidden (Krebs et al. 1996). Researchers have demonstrated superior spatial memory in several such species when compared with close relatives that do not rely heavily on remembering locations of stored food (e.g. Kamil et al. 1994). In addition, one part of the forebrain, the avian hippocampal complex (hereafter: hippocampus), is larger in food-storing species, presumably because of natural selection for better spatial memory (e.g. Hampton et al. 1995).

In the present study we compared the spatial memory and hippocampus of two subspecies of dark-eyed junco that differ primarily in their migratory behaviour. One

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subspecies, *J. h. hyemalis*, migrates thousands of kilometres each year and the other subspecies, *J. h. carolinensis*, is entirely sedentary or moves only a few kilometres between nesting territories and wintering home ranges. Both subspecies spend the winter together in mixed flocks within the year-round range of the nonmigratory birds, providing an opportunity for a natural experiment in which we could control for environmental influences other than those occurring when the migrants travel north to breed in the spring. By using two subspecies that may have split as recently as the last glaciation (~10 000 years; G. Barrowclough, personal communication) and matching subjects for age, sex, geographical location and capture time, we minimized many of the extraneous variables and differences in evolutionary histories that have plagued earlier comparative studies of behaviour and brain.

Our general objective was to determine whether the evolution of migratory behaviour in juncos led to cognitive and neuroanatomical changes as well. Our specific objective in experiment 1 was to determine whether migrant juncos performed better on tests requiring memory for spatial information. We used

several versions of our memory test and required birds to remember information for a range of retention intervals from minutes to hours. Our specific objective in experiment 2 was to identify the possible neuroanatomical basis for any advantage in spatial memory demonstrated by migrant juncos in experiment 1. We examined the hippocampuses of migrant and nonmigrant juncos for differences in neuron density, cross-sectional area of the cell bodies of these neurons relative to the area of the hippocampus, and hippocampal volume relative to that of the rest of the forebrain.

STUDY SPECIES

Junco h. hyemalis (hereafter migrants) breeds in hardwood forests, and primarily coniferous forests, across Canada and the northeastern U.S.A. *Junco h. carolinensis* (hereafter nonmigrants) breeds in pockets of glacial relict northern hardwood and coniferous forest above elevations of 800 m in the southern Appalachian mountains. In autumn, migrant juncos move south and occupy a wide range of habitats in the southern and central U.S.A. Among the nonmigrants, some individuals remain on their breeding territories throughout the winter and others move to lower elevations a few kilometres from their nesting sites or farther (Rabenold & Rabenold 1985). We captured and individually marked nonmigrants on their breeding territories in the summer. In January, we classified all marked birds recaptured within 0.5 km of their nest site as sedentary and all birds captured below 800 m as wandering nonmigrants. In three cases wanderers had been marked during the breeding season 4–6 km away, but most were unmarked.

The two subspecies differ in subtle morphological characters that can be distinguished in the hand by experienced observers. Migrants have a pink-tinted bill and nonmigrants have an ivory/blue-tinted bill. Migrants also have a shorter upper surface of the bill and males are smaller and darker than male nonmigrants. All subspecific classification was confirmed by two observers (D.A.C., C.W.S.Z.). We determined sex by plumage colour and wing length, and classified age as either young-of-the-year (hereafter 'young') or older (hereafter 'adult') by eye colour, skull ossification and plumage (Ketterson & Nolan 1976; Pyle 1997). Both subspecies show pronounced sex-based differences in movement during the nonbreeding season, such that male migrants range less than females (Ketterson & Nolan 1976) and male nonmigrants are less likely to leave their breeding territories than are female nonmigrants (C. W. S. Ziegenfus, unpublished data). Therefore, to reduce variance, we limited our study to males. In experiment 1, we used both young and adult males for behavioural studies. In experiment 2, we used only adult males to reduce variance in brain measurements and thereby increase statistical power. Although age cannot be accurately determined beyond 1 year in juncos, we suspect that very few individuals live beyond 2–3 years. Thus, there was probably little variation in age within the adult age class.

EXPERIMENT 1

In experiment 1, we tested the prediction that a migrant bird is better able to remember spatial information than is a nonmigrant. The magnitude of the spatial and temporal scales of the information to be remembered in our experiment was necessarily much less than that relevant to migratory journeys. We used a room-sized spatial matching-to-sample (sometimes called a one-trial associative memory) test, in which locations visited once had to be remembered for minutes to hours. In contrast, our migrant study species travels hundreds or thousands of kilometres and relocates breeding territories after being away for many months. None the less, we predicted that if there has been natural selection for enhanced spatial memory in migrants, it would require modification of the neural substrate for memory and may have resulted in enhanced spatial memory at various scales. For example, spatial memory tests, using small operant chambers with short memory intervals, have shown that bird species that rely heavily on recovering stored food after many months within extensive home ranges outperform related species that rely less on stored food (Balda et al. 1996). We conducted experiment 1 over two winters. In the second year we made a few methodological changes, as noted below, to accelerate training and testing. Despite changes in methodology, we combined the results from both years, because we detected no difference between years.

Methods

Husbandry

We captured 63 juncos on 11 December 1999, 5 November 2000 and 17 January 2001 in Giles County, Virginia, U.S.A. near Mountain Lake Biological Station. The birds were transported to Williamsburg, Virginia and divided into test flocks, each flock containing two migrant and two nonmigrant individuals. After being placed in flocks, birds were assigned numbers and thereafter their subspecies designation was either ignored (senior author) or unknown (all other authors and technicians) throughout the experiment. Flocks were housed in outdoor, wire-mesh cages measuring $3.1 \times 2.4 \times 2.1$ m (height). Each cage contained one cut conifer tree for roosting, one artificial tree made of dowels, four natural tree branch perches, one water dish and four food trays.

Birds were supplied with vitamin-enriched water and a seed mixture containing red and white millet, cracked corn, thistle, sunflower seed, turkey starter mash with antibiotics and oyster/sand grit. Food and water were available ad libitum except for periods of food deprivation. Deprivation was begun in the afternoon of the day before birds were to be tested. The exact time that deprivation was initiated depended on the temperature, with shorter deprivation periods on colder days. We monitored subcutaneous fat reserves of all individuals daily to ensure that the birds were not at risk of starvation during periods of deprivation. Although natural temperature fluctuations may have resulted in varying levels of motivation, our design, in which two migrants and two nonmigrants were housed in the same cage, deprived

together and then tested simultaneously in different test rooms, should have minimized any effect of motivation on the comparison between migrants and nonmigrants. In 2000, we began deprivation at daybreak on the day of testing, rather than on the afternoon before testing. However, birds were generally tested later in the day in 2000, so total deprivation time was similar to that in 2001.

Training

After 1 week in captivity we acclimated each test flock for 2 h/day for 1 week in test rooms. Test rooms measured approximately 4.5 m², with 2.7-m ceilings, and were similar in appearance except that the position of the windows, heaters and electrical outlets varied. One artificial tree was present in each test room. Other potential perches were covered with plastic so that all birds learned to start tests from the artificial tree perch. Five feeders, fashioned from metal containers (10 × 7 × 7 cm), were present on the floor during acclimation. Using coloured paper tape, we created 97 unique feeders (and five identical white feeders). Feeder diversity was increased further by placing trial-unique objects on the tops of the feeders, such as toy figurines or rocks. A 4 × 4-cm opening was cut in the plastic lids of the containers to make the food accessible. This opening could be covered by removable, yellow adhesive 'post-it'-style paper notes. As acclimation proceeded we reduced the number of feeders containing food. By the end of the first week, most birds were checking the feeders consistently. We then began training the birds singly, rotating them through the four test rooms so that each bird was trained or tested in each room at least once each week. The order in which birds were run each day was rotated to equalize food-deprivation levels across flocks.

The goal of the first stage of training was to teach the birds (1) to remove adhesive covers from the feeders, (2) that only one of the five feeders in each trial would ever contain a reward, and (3) that each trial was a unique event with no relationship to past or future trials. We began with three of the five feeders rewarded, and the adhesive cover on all feeders obstructing only 25% of the opening. If the bird obtained any food reward within 10 min, it passed the trial and proceeded to the next stage of training. If it failed, it was given the same training trial with a new feeder arrangement the next time it trained, and this continued until it acquired a reward. Food was restricted to one feeder after two to three trials and the adhesive cover was progressively lowered until birds had to remove it to see and eat the reward. To avoid the possibility that birds would associate certain feeders with rewards, no bird ever saw the same set of five feeders more than once throughout the study. Each training trial had a specific set of feeders associated with it so that all birds progressed through the same series of feeders until they reached criterion for testing. In all training trials, feeders were placed in the same five positions on the floor. Rewarded feeders were randomly selected and a reward consisted of six mealworm halves. We trained or tested birds daily in 2000. In 2001, we trained and tested birds every other day to minimize carryover from previous

tests. When a subject acquired a reward on three successive training trials it was moved on to the second stage of training.

The goals of the second stage of training, called pretesting, were to (1) introduce a time interval requiring memory into the trials and (2) determine whether birds had learned the test procedure, as demonstrated by reaching our below-chance performance criterion. In phase 1 of pretesting, birds were allowed to check the feeders by removing the adhesive covers and to eat one piece of mealworm from the rewarded feeder. Before they could consume the additional five mealworm pieces we interrupted them by walking into the room, causing them to fly up to the perch, thus initiating a retention interval (RI). During the retention interval the subject was either left on the perch with the lights out (2000) or captured and placed into a darkened container (2001), thus preventing the updating of memory. During the RI, we replaced the adhesive cover on the feeders that the bird had checked. After the RI, in phase 2, the birds demonstrated their spatial memory ability by returning to the rewarded feeder. Pretesting progressed in six steps, with RI ranging from 30 s to 10 min. In both phases 1 and 2, the birds were given 10 min to find the food reward. If the reward was not found in 10 min, the pretest ended as a failure and the same pretest, with new feeders, was given on the next training trial.

The final pretest mimicked what the bird would face during testing. The criterion for moving on to testing was to find the baited feeder on the first or second attempt on three consecutive versions of the pretest. Because the likelihood of passing three tests consecutively by chance was approximately 5%, our criterion ensured that birds had learned to perform the actions required for testing and were not simply checking feeders at random in phase 2. In 2000, the longest RI used in training was 90 s and the criterion for moving on to testing was slightly more stringent ($P < 0.03$).

Testing

The testing regimen consisted of six tests: four versions, with varying RIs, in which both spatial location and colour predicted the reward (space/colour tests), one in which spatial location but not colour predicted reward (space only test) and one in which spatial location and colour were dissociated (dissociation test). In space/colour tests, each feeder had a different colour, pattern and object, providing cues in addition to spatial location that might be helpful in relocating rewarded feeders (short space/colour: RI=1.5 min; medium space/colour: RI=10 min; long space/colour: RI=60 min; longest space/colour: RI=120 min). Feeders were never reused in later tests, so every test array was unique. In space-only tests, identical white feeders were used, so only spatial information was available and all tests were the same except for the location of the reward (RI=1.5 min in 2000, 10 min in 2001). The dissociation test began like a space/colour test, but before phase 2, we swapped the rewarded feeder and a randomly selected nonrewarded feeder, forcing the birds to choose between making their first visit to the rewarded feeder or to the previously rewarded location

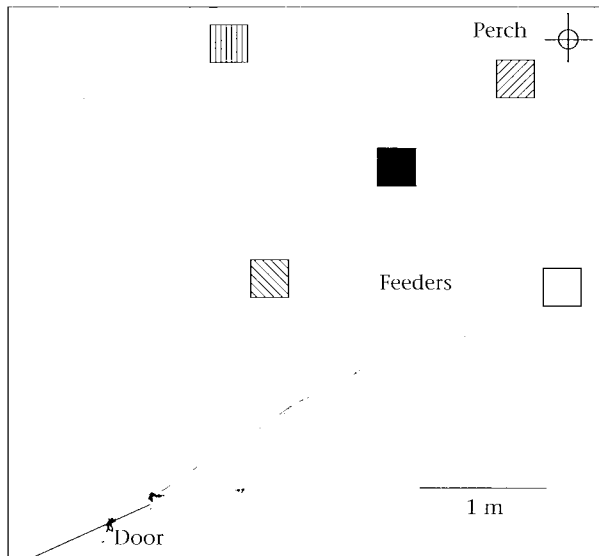


Figure 1. Schematic of testing room showing typical positions of feeders during tests.

(which now contained a different feeder) (RI=2 min in 2000, 10 min in 2001). During phase 2 of the dissociation test, we removed the remaining five pieces of mealworm to encourage the birds to continue checking the feeders, thereby allowing us to see where subjects looked after they found their first-choice feeder empty.

During testing, we altered the spatial arrangement of the feeders from that during training to reduce the chance of carryover of any habitual patterns of checking feeders. Like the arrangement used during training, the testing arrangement minimized the possibility of trap-lining, because after a bird checked one feeder, there were several others equidistant (Fig. 1). In both phases of all tests, we recorded, as an index of motivation, each bird's latency to leave its perch and begin checking the feeders. Our most important dependent variable was the number of visits required to return to the baited feeder, with one visit being the best performance possible, and an average of three visits being the expectation for random search.

In 2001, after reaching criterion, birds received one trial each of medium space/colour, space-only and longer space/colour tests, and this sequence was repeated two more times. Then birds received one trial of longest space/colour and completed testing with a nonrewarded dissociation test of space/colour. In 2000, the order of testing was as follows: three short space/colour tests followed by three medium space/colour tests. Four dissociation tests were given in 2000, and because dissociations were unrewarded, each was followed by a series of six short space/colour tests to prevent extinction through lack of reward. A unique set of feeders was used for each version of each test, but the same feeders were used each time the same test was administered to a different bird.

In 2000, the arrangement of feeders on the floor was changed between tests, so that no two tests were the same but all birds received the same series of arrangements. In 2001, we used the same arrangement of feeders on the

floor for all tests to reduce variance in performance arising from apparent preferences for certain parts of the test rooms.

The maximum time allowed in phases 1 and 2 was 10 min. If a bird did not locate the food reward within 10 min (i.e. because it never left its perch), it was classified as a failure. If a bird failed a test, it continued to progress through the sequence until it had completed all but the dissociation test, at which point the failed tests were readministered.

All birds were released at their site of capture soon after they completed testing. Most subjects were returned to the wild before migration or breeding began. Four birds suffered accidental deaths during capture or transport, but none were harmed as a result of food deprivation, training or testing.

Statistical analyses

To evaluate each subject's performance, we calculated a mean score for all replicates of that test. We then combined the results of the medium space/colour and space-only tests into one overall measure of performance for statistical testing, because we had no a priori reason to predict differential performance of the subspecies on the different types of tests. To determine whether all subjects combined achieved better-than-chance performance on each type of test, we used equations for sampling a fixed number of sites without replacement (Tillé et al. 1996). Because the criterion for chance performance varies with sample size, we also provide the maximum below-chance performance level for each test ($\alpha=0.05$) for comparison.

For comparisons between subspecies, we used a Wilcoxon two-sample test, a nonparametric equivalent to the *t* test, because in a few cases, sample sizes were unequal or variance was heterogenous. Overall performance on space-only tests and dissociation tests did not differ significantly between 2000 and 2001, despite different RIs, so we combined these results to achieve greater statistical power. Power was calculated for all nonsignificant comparisons from which conclusions were drawn using a hypothesized 'medium' effect size ($d=0.6$; Cohen 1988) and the relative power efficiency of the Wilcoxon two-sample test (0.97; Siegel & Castellan 1988).

We compared acquisition across subspecies by determining, separately for phases 1 and 2 of training, the number of days to reach criterion for each bird relative to the maximum number of days to reach criterion for any bird. Thus, a bird that took half as long as the slowest bird to reach criterion in phase 2 received an acquisition score of 0.5 for that stage. We then compared the mean acquisition scores for both subspecies.

Results

Considering all subjects, performance was significantly better than that expected for random search on the short, medium and long space/colour tests (Fig. 2) and space-only tests (Fig. 3). On the space/colour tests, more visits were required to return to the rewarded feeder when subjects had to remember the information for longer,

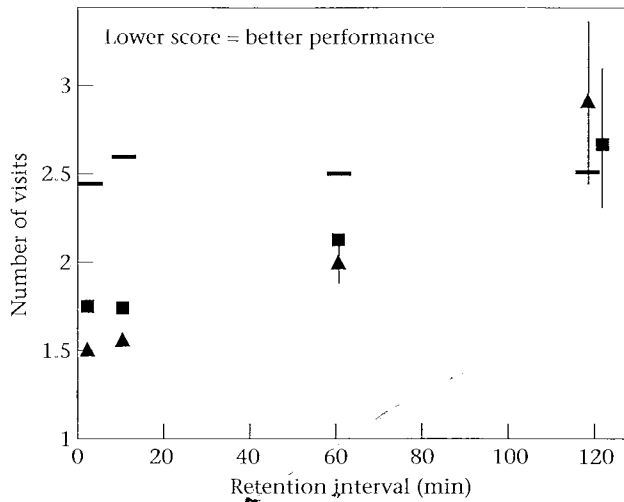


Figure 2. Performance of migrant (\blacktriangle) and nonmigrant (\blacksquare) dark-eyed juncos on tests of spatial memory in which both spatial and feeder-specific cues were available. Points indicate the mean (\pm SE >0.18) number of visits required to return to the rewarded feeder after the indicated retention interval. The maximum number of visits that would have been significantly below that expected for a randomly searching bird is shown for each test (—). See text for statistical analyses.

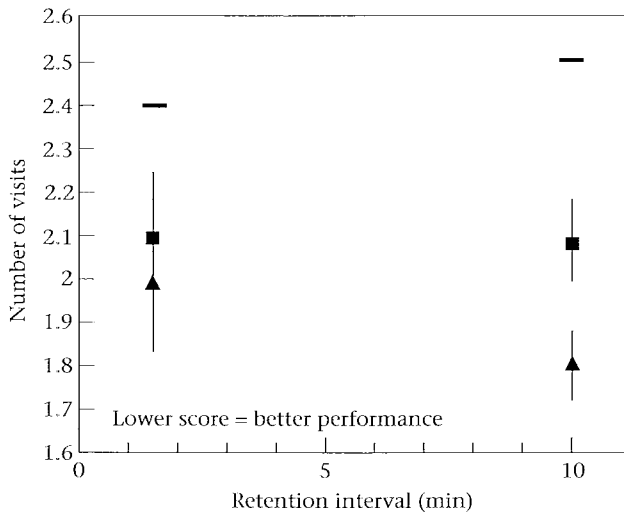


Figure 3. Performance of migrant (\blacktriangle) and nonmigrant (\blacksquare) dark-eyed juncos on tests of spatial memory in which only spatial cues were available. Points indicate the mean \pm SE number of visits required to return to the rewarded feeder after the indicated retention interval. The maximum number of visits that would have been significantly below that expected for a randomly searching bird is shown for each test (—). See text for statistical analyses.

such that on the longest space/colour test, performance had declined to below chance (Fig. 2).

Migrant juncos performed significantly better than nonmigrants on the overall combined mean of the medium space/colour and space-only tests ($\bar{X} \pm$ SE: migrants: 1.64 ± 0.49 visits; nonmigrants: 1.92 ± 0.46 visits; Wilcoxon two-sample test: $Z=1.97$, $N=41$, $P=0.0485$; see Figs 2, 3 for individual means). There were no

significant differences in subspecies' performance on either the short or the long space/colour tests, but power was low (Wilcoxon two-sample test: short: $Z=0.75$, $N=16$, $P=0.45$, estimated a priori power=0.20; long: $Z=0.34$, $N=26$, $P=0.73$, estimated a priori power=0.30; Fig. 2); thus, any conclusion from these two comparisons must be tentative. Neither species performed significantly better than chance on the longest space/colour test, so we did not test for differences between subspecies (Fig. 2).

When the rewarded feeder was moved to a different spatial location in phase 2 of the dissociation test, birds usually visited the correct spatial location first ($X \pm$ SE: correct space: 1.63 ± 0.74 visits; correct feeder: 2.69 ± 1.05 visits; $N=46$). Their preference for visiting a previous spatial location before a previously rewarded feeder was highly significant (paired t test: $t_{45}=5.34$, $P<0.0001$). When birds found the first feeder empty, they usually continued checking feeders, and those that did were more likely than expected by chance to make their next visit to the previously baited feeder (using Tillé et al.'s 1996 equation for sampling without replacement for all 38 birds that visited the correct spatial location on their first visit: $P=0.003$). Subspecies did not differ in number of visits required to find the correct spatial location during dissociations ($X \pm$ SE: migrant: 1.76 ± 0.75 , $N=21$; nonmigrant: 1.53 ± 0.73 , $N=25$, Wilcoxon two-sample test: $Z=1.16$, $P=0.25$) or the correct feeder (migrant: 2.72 ± 1.11 , $N=21$; nonmigrant: 2.65 ± 0.98 , $N=25$; $t_{44}=1.036$, $P=0.31$). However, because the sample size of 46 individuals provided low statistical power for both of these tests (a priori estimate=0.50), the conclusion that subspecies did not differ on the dissociation tests remains tentative.

There were no apparent differences between subspecies in acquisition or motivation. There were no subspecific differences in the proportions of birds failing during either phase of training: of 31 migrants and 32 nonmigrants that began training, 16% of migrants and 13% of nonmigrants failed to graduate from phase 1, and of these, 11% of each subspecies failed to reach criterion during phase 2 of training (Fisher's exact tests: NS). Among birds that reached criterion for testing, there were no apparent subspecific differences in acquisition scores ($\bar{X} \pm$ SE: phase 1: migrant: 0.51 ± 0.23 , $N=24$; nonmigrant: 0.48 ± 0.22 , $N=28$; Wilcoxon two-sample test: $Z=0.50$, $P=0.62$; phase 2: migrant: 0.45 ± 0.22 , $N=23$; nonmigrant: 0.49 ± 0.23 , $N=27$; $Z=0.10$, $P=0.92$). There was no indication of a subspecific difference in motivation as measured by latency to start searching ($X \pm$ SE: migrant: 75.3 ± 56.8 s, $N=18$; nonmigrant: 75.7 ± 60.1 s, $N=23$; Wilcoxon two-sample test: $Z=0.07$, $P=0.94$), so any effect of motivation on performance would have been random with respect to the comparison between migrants and nonmigrants. Additionally, there was no relationship between motivation, as measured by latency to begin searching for food in phase 2 of the test, and performance, as measured by the overall combined mean performances on medium space/colour and space-only tests (linear regression: performance= 0.001 latency $+1.87$, $R^2=0.01$). With respect to age, there was no evidence to suggest a difference in performance, as measured by the overall combined mean performances on medium

space/colour and space-only tests ($\bar{X} \pm \text{SE}$: young: 1.82 ± 0.45 ; adult: 1.81 ± 0.51 ; $t_{38}=0.10$, $P=0.92$).

Discussion

Juncos performed better than chance on the spatial memory tests, but performance declined as RI increased from 1.5 to 60 min, and when birds waited 120 min, they no longer returned to rewarded feeders significantly more often than expected by chance. This suggests that our range of tests included tests of appropriate difficulty for this species.

Migrants outperformed nonmigrants when comparing the overall mean of space/colour and space-only tests with a medium RI (10 min). On a longer version (RI=1 h) of the space/colour test, performance declined and there was no significant difference between subspecies, but because only 26 birds completed this test, statistical power was too low to warrant a firm conclusion. Neither subspecies performed significantly better than chance after the longest RI (2 h). Ceiling effects may have contributed to the smaller differences on longer tests, which may have been too difficult for the subjects to reveal differences between migrants and nonmigrants.

We expected juncos to be attuned to both feeder-specific cues, such as colour patterns, as well as spatial cues, based on evidence from two previous studies of dissociation of cues using migrant juncos on a similar room-scale spatial memory test or an operant chamber test (Brodbeck 1994; Brodbeck & Shettleworth 1995). However, in our dissociation experiment, both subspecies preferentially returned to the previously rewarded spatial location rather than showing no preference between previously rewarded feeders and locations as reported by others. Although these previous experiments used birds of the same subspecies as our migrants, they differed from our experiment in several ways. Specifically, both of these previous experiments used a smaller sample of birds (3–4 individuals) and retested each individual repeatedly using either movable testing arrays or touch-screen monitors. In our test, juncos fed on the ground, which is their typical foraging behaviour, and we tested 20–25 individuals of each subspecies. A preference for spatial cues over feeder-specific cues, like that demonstrated in our experiment, has been reported for several food-caching birds and has been interpreted as evidence of a cognitive adaptation for accurate memory of cache locations (Clayton & Krebs 1994a; Brodbeck & Shettleworth 1995). Although our dissociation test was fundamentally similar to previous tests of dissociation of cues using juncos, the juncos in our study appeared to rely heavily on spatial cues to locate rewards, a behaviour thought to be more characteristic of food-storing birds (Brodbeck 1994). Our finding, that juncos, which never store food, also had a strong preference for spatial cues over feeder-specific cues, raises the question of the generality of previous reports that food-storing and non-storing birds have qualitative differences in cognitive processing.

EXPERIMENT 2

The well-studied mammalian hippocampus is intimately involved in spatial memory, and hippocampal morphology corresponds to differences in ecological space use between mammal species and between sexes within some mammal species (reviewed in Lavenex et al. 2000). The less well-studied avian hippocampus appears to be functionally and anatomically homologous to the mammalian hippocampus (reviewed in Colombo & Broadbent 2000). The role of the avian hippocampus in spatial memory has been demonstrated experimentally in the context of memory for locations of stored food over periods of minutes to months (Hampton & Shettleworth 1996, and references therein). It is also supported by comparisons of bird species that do and do not store food; food-storers generally have greater hippocampal volume (Krebs et al. 1989; Sherry et al. 1989; Basil et al. 1996; but see Volman et al. 1997), and in at least one comparison, longer-lasting spatial memory (Biegler et al. 2001). Additionally, hippocampal morphology may be affected, even within the lifetime of a bird, by ecological factors such as season (Barnea & Nottebohm 1994; Smulders et al. 1995), complexity of nesting habitat (Abbott et al. 1999), need to remember nest sites (Sherry et al. 1993), and even confinement in small cages (Smulders et al. 2000).

Surprisingly, there is, so far, little evidence that the hippocampus is involved in the most extraordinary feat of space use accomplished by birds, their repeated migrations over distances of hundreds to many thousands of kilometres. In homing pigeons, *Columba livia*, which are not migrants but will return to familiar home areas when released, hippocampal lesioning impairs recognition of the familiar home area and prolongs length of the return trip, but does not affect the initial bearing taken on the homing journey (e.g. Bingman & Mench 1990; Strasser et al. 1998). There is also suggestive evidence that a migratory (*Sylvia borin*) and a nonmigratory (*S. melanocephala*) warbler differ in hippocampus size: the long-distance migrant has a relatively large hippocampus after experiencing migration, whereas the nonmigrant shows no such tendency (Healy et al. 1996). However, the difference in relative hippocampal volume between these warbler species was largely due to the reduction in the size of the rest of the forebrain, rather than an absolute increase in size of the hippocampus, so an interpretation is not straightforward. In addition, the amount of local movement or short-distance migration characteristic of the nonmigrant warbler species was not known, and the effect of captivity on hippocampal volume could explain some of the results.

While it makes sense that the hippocampus, involved as it is with spatial memory, would be associated with migratory behaviour in birds, there are also reasons to think that it might not be associated with this behaviour. First, two correlative studies across avian families failed to find a relationship between migratory behaviour and hippocampal volume (Sherry et al. 1989; Healy et al. 1991). Second, there is a large unlearned component to migration in many bird species

that could function without experience-based memory, as evidenced by the remarkable solo flights of naïve young birds, and the demonstrated genetic basis for migratory orientation and restlessness (e.g. Berthold & Querner 1981).

The results of experiment 1 indicated that migrant juncos had better spatial memory than nonmigrants on a room-scale test. This is consistent with the hypothesis that the selective advantage of accurate relocation of breeding sites and wintering home ranges has led to enhanced spatial memory in migrants. The primary objective of experiment 2 was to compare the hippocampus, which has been shown to have a role in spatial memory (e.g. Hampton & Shettleworth 1996), in migratory and nonmigratory juncos to test our prediction that migratory birds would have a greater physiological investment in their hippocampuses. We made three comparisons to determine whether the migrant subspecies invests more heavily in hippocampus than the nonmigrant subspecies. First, we calculated the density of hippocampal neurons in each subject's brain to determine whether migrants and nonmigrants differed in their investment in hippocampal nerve cells. Second, we examined the size of the cell bodies of hippocampal neurons, relative to the size of the hippocampus, under the assumption that larger cell bodies would correspond to more projecting axons, greater connectivity and superior spatial memory ability. Finally, we compared overall hippocampal volume, relative to the rest of the forebrain, as a way of including any non-neuronal differences (e.g. glial cell proliferation).

Any difference in hippocampus that we might detect between the recently evolved migrant and nonmigrant junco subspecies is likely to be related to migration behaviour itself, or some difference resulting from a migratory lifestyle. One attractive explanation is that the migratory journey relies extensively on spatial memory and thus requires or leads to a larger hippocampus. An alternative explanation is that migration itself is not related to a larger hippocampus, but to the fact that migrants occupy and must familiarize themselves with at least two home ranges each year, as opposed to one for nonmigrants. Because space use varies among individuals of the nonmigratory junco subspecies, our natural experiment allowed us to begin to distinguish between these two alternative explanations for hippocampal differences between migrants and nonmigrants. Some nonmigrants remain on the same small territory year-round and others choose new home ranges in winter just like migrants.

Methods

Histological preparation

We captured 49 adult male juncos on 18–26 December 1997, 15–30 January 1999 and 6–8 January 2000 between 0700 and 1300 hours using mist nets baited with grain at the same location described for experiment 1. Within 1–3 h of capture, they were transported to a field laboratory and killed using an overdose of anaesthetic (Ketamine/Xylazine at a 17:1 ratio injected

intramuscularly at 0.005 ml/g). The birds experienced no pain beyond that of a brief intramuscular injection. Birds were then perfused transcardially with saline followed by 10% paraformaldehyde for 10 min. Brains were removed from the skull and postfixed in 10% paraformaldehyde for 1–24 h, depending on the quality of the initial perfusion. Brains were then stored at 5°C in saline preserved with sodium azide for 30–60 days. Tissue was cryoprotected in 30% sucrose for 24 h before sectioning and then frozen with dry ice and sectioned in a coronal plane at 40 µm in a cryostat. We mounted sections on gelatin-coated slides and stained them with cresyl violet.

Quantification

We quantified neuron number and cell body area, as well as volume of the hippocampus and remainder of the telencephalon, using NIH Image software on two Power Macintosh computers equipped with scope-mounted video cameras. For both counting and measuring hippocampal neurons, we sampled four consistent locations along the rostral–caudal axis, based on four distinct anatomical features (appearance of nucleus basalis, invagination of the ventricle near the ventral surface of the telencephalon, appearance of commissura anterior, and appearance of darkly staining cerebellum). The four sampled sections correspond approximately to depth positions A 5.0, A 3.0, A 1.6 and A 0.8 in Stokes et al. (1996).

To estimate the number of neurons in the hippocampus, we divided each hemisphere into dorsolateral, dorsomedial and ventral subfields following Healy et al. (1994), and counted all neurons in a single microscope field ($0.1426 \times 0.0845 \text{ mm}^2$) near the centre of each hippocampal subfield using a $40 \times$ objective with a $0.6 \times$ video coupler (final magnification $24 \times$). Neurons were identified by visible nucleolus and large size relative to glial cells, and only those in focus were counted. This sampling protocol resulted in the inclusion of approximately 40 neurons per microscope field, three fields per hemisphere per section, and four sections per brain, or approximately 960 neurons per bird. We calculated neuron density separately for each subfield (averaged across hemispheres and rostral–caudal positions) by dividing neuron number by tissue volume (field area \times section width).

We used a higher magnification to estimate the size of neurons ($40 \times$ objective and $2 \times$ camera coupler for $80 \times$ final magnification). We quantified the area of the cell body of every cell in the centre of each of the three hippocampal subfields on the same sections of each hemisphere used to count neurons. If fewer than 10 neurons were present in the first field, all of those in an adjacent field were measured as well, until 10 or more had been measured. We then randomly selected 10 measurements for inclusion in the data set. This resulted in 240 cell body areas per bird. We determined mean neuronal cell body area separately for each hippocampal subfield by averaging across hemispheres and rostral–caudal positions.

To calculate relative hippocampal volume, we measured the area of the hippocampus and the

remainder of the telencephalon on every fourth section. Telencephalon measurement commenced with the appearance of the first traces of hippocampus, and continued caudally until the last appearance of the hippocampus. The hippocampus was measured from its first appearance until the last section in which it was visible (often the caudal-most section acquired). Boundaries of the avian hippocampus are quite distinct with the exception of the short lateral border in sections intermediate along the rostral–caudal axis.

A few caudal sections in about half of the brains were missing due to problems with cutting and mounting such small sections. When sections that had contained hippocampus were missing, we estimated the areas of both telencephalon and hippocampus on those sections using the following procedure: (1) calculate the rate of diminution for telencephalon or hippocampus using the previous five sections in that hemisphere, (2) estimate the area of each missing section based on this function, (3) cease adding estimated sections when the area of the hippocampus declined to 14.4% of the largest hippocampal area for that brain, which was the average proportion for the last section in the 26 complete brains in the study. We used this technique to estimate missing sections for 23 of the brains in the study, thereby adding an average \pm SE of $2.6 \pm 1.8\%$ to the volume of each adjusted brain.

Statistical analyses

To minimize any effects of between-year or between-observer bias in our comparison of the two subspecies, we sampled individuals of both subspecies during each day of capture. We stained tissue from all birds collected within each year in a single batch and we used the same cryostat for sectioning across all 3 years of the study. Birds collected within the same year were also measured by the same observer using the same computer for any given dependent variable. Thus, any bias or inconsistency would produce random error with respect to the comparison between subspecies. After brains were removed from the birds, we assigned codes to each sample and performed all histology and quantification entirely blind to subspecies identity. We compared non-normally distributed variables with a nonparametric Wilcoxon two-sample test. Because of consistent treatment group differences in forebrain size (see below), we controlled for size in two of our three main comparisons using analysis of covariance (ANCOVA): (1) for cell body areas using total hippocampal area as a covariate; and (2) for hippocampal volume using the volume of the rest of the telencephalon as a covariate. Because our objective in this experiment was to compare two subspecies, we did not use corrections that involved multiplying by constants, such as for split nucleoli or overcounting, because these would not affect the comparison between subspecies.

Although our sample sizes were larger than those used in nearly all studies of avian hippocampus ($N=28$ for between-subspecies comparisons; $N=20$ for within-subspecies comparisons), many of which have produced significant results, they were not large enough to allow reliable conclusions based on negative results (Cohen 1988). For comparisons between subspecies, where large

differences might be expected, the a priori estimate of power was approximately 0.50 for t tests and somewhat higher for ANCOVAs. Despite this, we present statistical analysis for these results, although our failure to find differences between groups under these circumstances is not strong evidence that no differences exist. For the within-subspecies comparisons, where smaller differences might be expected, the a priori estimate of power was approximately 0.20–0.30, making any conclusions based on negative results unreliable. Therefore, in these cases, we present only the means and do not make any conclusions.

Results

Migrants versus nonmigrants

Migrant juncos had slightly but significantly smaller wing length, body mass upon capture, mass of fixed brain, tarsus length (i.e. foot bone) and telencephalon volume (excluding the hippocampal portion) than nonmigrants (Table 1).

Migrant juncos had significantly more densely packed hippocampal neurons overall ($\bar{X} \pm$ SE: migrant: $92.9 \pm 12.4 \times 10^3/\text{mm}^3$; nonmigrant: $82.2 \pm 14.9 \times 10^3/\text{mm}^3$; $t_{26}=2.06$, $P=0.05$). Although this difference was significant for all subfields combined, when each subfield was considered alone, only the difference in neuron density in the dorsolateral subfield was significant (Table 1).

The absolute cell body areas of hippocampal neurons of migrant juncos were smaller than those of nonmigrants when all hippocampal subfields were combined ($\bar{X} \pm$ SE: migrant: $118.8 \pm 17.8 \mu\text{m}^2$; nonmigrant: $135.9 \pm 18.3 \mu\text{m}^2$; $t_{26}=2.5$, $P=0.02$), but not for any subfield separately (Table 1). To correct for the significant difference in overall brain size between subspecies, average cell body area was predicted from the confounding variable of hippocampal area (sum of area of all sections), and then the variable of interest (subspecies) was added to the model (ANCOVA: subspecies: $F_{1,24}=0.57$, $P=0.46$; hippocampal area: $F_{1,24}=16.4$, $P=0.0005$; interaction: NS). Individual birds with larger hippocampal areas had larger neuronal cell bodies, but we found no evidence to suggest that neuron size was related to subspecies.

We found no evidence of hippocampal volume differences between migrants and nonmigrants, contrary to our prediction. This result held for either absolute differences (Table 1) or the more relevant analysis that statistically controlled for the significant difference in telencephalon volumes between the two subspecies (ANCOVA: subspecies: $F_{1,24}=0.58$, $P=0.45$; telencephalon volume: $F_{1,24}=44.3$, $P=0.0001$; interaction: NS). Thus, while hippocampal volume was highly correlated with the volume of the rest of the telencephalon, we found no evidence to suggest that hippocampal volume differed between subspecies when we statistically controlled for overall forebrain differences. However, we remain tentative about this conclusion, because our sample of 14 individuals from each subspecies provided low statistical power (a priori estimate=0.50–0.60; Cohen 1988).

Table 1. Measurements of migrant and nonmigrant juncos uncorrected for size differences

	Migrant ($\bar{X}\pm\text{SD}$)	Nonmigrant ($\bar{X}\pm\text{SD}$)	<i>t</i>	<i>P</i> *
Wing length (mm)	82.1±1.5	84.9±1.7	4.56	0.0001
Body mass (g)	20.5±1.3	24.0±1.6	6.36	0.0001
Brain mass (g)	0.73±0.05	0.80±0.07	2.57	0.02
Tarsus length (g)	20.9±0.5	22.0±0.7	4.37	0.0002
Telencephalon volume (mm ³)	296.1±35.4	334.9±45.3	2.52	0.02
Hippocampal volume (mm ³)	12.7±1.9	14.3±2.6	1.69†	0.11
Neuron area (μm ²)				
Dorsolateral	120.48±19.83	132.13±17.41	1.53	0.14
Dorsomedial	123.10±19.80	132.94±16.22	1.33	0.20
Ventral	118.03±16.68	131.06±18.46	1.82	0.08
Neuron density (×10 ³ /mm ³)				
Dorsolateral	95.2±14.5	82.5±14.0	2.35	0.03
Dorsomedial	88.8±13.2	79.2±14.4	1.85	0.08
Ventral	94.8±12.4	84.9±18.4	2.66	0.11

*Sample size for all comparisons was 14 migrant and 14 nonmigrant juncos.

†Wilcoxon two-sample test used due to unequal variances. Number indicated is Z score.

Sedentary versus wandering nonmigrants

Sedentary nonmigrants were heavier than wandering nonmigrants, and possessed heavier brains (body: $t=2.78$, $P=0.01$; brain: $t=3.04$, $P=0.007$; Table 2). Because of these unexpected differences in body and brain mass, we used the same size corrections as we did when comparing subspecies.

We calculated the same measurements for sedentary and wandering birds as for migrants and nonmigrants, but because of the smaller sample sizes (10 of each), expected statistical power was too low to allow for meaningful statistical comparisons. The mean \pm SE neuron densities for all subfields combined was $84.1 \pm 8.7 \times 10^3/\text{mm}^3$ for sedentary birds and $82.9 \pm 6.4 \times 10^3/\text{mm}^3$ for wandering birds (see Table 2 for individual subfield means). The absolute cell body area of the hippocampal neurons, averaged across the three subfields, was

$137.8 \pm 6.4 \mu\text{m}^2$ for sedentary birds and $130.5 \pm 10.5 \mu\text{m}^2$ for wandering birds (see Table 2 for individual subfield means). We also calculated the absolute hippocampal volume (Table 2) and the more relevant proportion of telencephalon volume comprised of hippocampus, which was $4.4 \pm 0.3\%$ for sedentary birds and $4.5 \pm 0.4\%$ for wanderers.

Discussion

We predicted that migrant birds would have greater physiological investment in hippocampus than nonmigrants, because our migrants outperformed nonmigrants on tests involving spatial memory in experiment 1, and because the avian hippocampus is involved in spatial memory. We measured hippocampal investment in three ways: (1) density of hippocampal neurons, (2) area of the neurons (diameter of cell body excluding axons and dendrites), and (3) volume of the hippocampus, which might reveal non-neuronal differences such as glial cell proliferation. Because treatment groups differed in the absolute sizes of their brains, we used an ANCOVA to hold brain size constant statistically. As predicted, migrants had more densely packed hippocampal neurons than nonmigrants. Contrary to our prediction, there was no indication that individual neurons of migrants were larger than those of the other subspecies; in fact, the means tended to differ in the direction opposite the prediction. Because the brains and bodies of migrants are smaller than those of nonmigrants, perhaps it is not surprising that their nerve cell bodies would not be larger. When nerve cell body size was compared with brain size controlled statistically, no difference was detected. A similar analysis of hippocampal volume relative to the volume of the rest of the forebrain revealed no detectable difference between migrants and nonmigrants. Interpretation will be difficult until more is known about the importance of neuron size, density and number in avian hippocampal function. A study on adult

Table 2. Measurements of sedentary and wandering juncos uncorrected for size differences*

	Sedentary ($\bar{X}\pm\text{SD}$)	Wanderer ($\bar{X}\pm\text{SD}$)
Wing length (mm)	84.1±1.5	84.7±1.8
Body mass (g)	24.1±1.5	22.6±0.8
Brain mass (g)	0.90±0.05	0.81±0.08
Tarsus length (g)	21.8±0.5	21.2±8.9
Telencephalon volume (mm ³)	359.7±26.4	313.6±63.7
Hippocampal volume (mm ³)	15.92±1.58	13.95±2.78
Neuron area (μm ²)		
Dorsolateral	132.1±9.5	124.8±13.8
Dorsomedial	142±6.7	135±10.1
Ventral	138.6±6.6	131.3±9.9
Neuron density (×10 ³ /mm ³)		
Dorsolateral	84.0±1.3	85.5±7.7
Dorsomedial	81.2±7.6	77.5±7.6
Ventral	87.1±9.7	85.8±7.0

*For all comparisons sample size was 10 sedentary and 10 wandering juncos.

and juvenile food-storing birds found greater hippocampal volumes in adults, but higher hippocampal neuron densities in juveniles (Healy et al. 1994). In another comparison, older individuals of a migratory warbler species had greater hippocampal volume, cell number and density than young birds of the same species that had not yet migrated, but there was no difference in neuron size (Healy et al. 1996). Unfortunately, most studies of avian hippocampal volume have not reported neuron size or density, so the effects of species ecology and individual experience on these variables are not well understood.

One explanation for the greater investment in hippocampal neuron density by migrant juncos is that it does not relate directly to the demands of the migratory journey, but rather results from the fact that migrants must familiarize themselves with two or more home ranges each year. Nonmigrant juncos either remain sedentary year-round, or wander from breeding territories to spend the winter on new home ranges at lower elevations, sometimes just a few kilometres from their breeding territories. By comparing sedentary and wandering nonmigrants, we attempted to determine whether establishing an additional home range, without undergoing migration, was sufficient to cause or require a modified hippocampus. The statistical power of these comparisons to detect differences would have been very low, so we did not attempt analysis (250 birds would have been required to achieve a reasonable power of 0.80). Thus, although there were no obvious differences in the mean values of brain variables for wandering and sedentary birds, we cannot say with confidence whether wandering and establishing multiple home ranges, in the absence of migration, causes or requires greater investment in hippocampal neurons. Our initial hypothesis was that the difference in hippocampus volume between migrant and nonmigrant juncos is related to different spatial memory requirements for each subspecies either during migration or during the breeding season when the two populations are apart. Because the nesting habitat of migrants and nonmigrants is generally similar (northern hardwood and conifer forests), we hypothesize that some aspect of the migratory journey results in greater investment of hippocampal neurons in migrants than in nonmigrants. However, we could not test the alternative hypothesis that establishing two home ranges is responsible for differences in hippocampus between migrants and nonmigrants. Thus, further research will be necessary to critically test this hypothesis.

GENERAL DISCUSSION

We took advantage of a natural experiment in which two very similar subspecies of dark-eyed juncos differ most notably in whether or not they migrate to a northern breeding area. Thus, we were able to ask whether a migratory lifestyle has led to enhanced spatial memory. We found that the migratory subspecies outperformed the nonmigrants on room-scale tests in which they were required to revisit locations where they had encountered food 10 min earlier. There were no apparent differences

in motivation or acquisition that could explain the better performance of the migrants. Thus, we supported our prediction that migrant juncos would have better spatial memory abilities than their nonmigratory conspecifics. This is not the first case in which an ecological difference relevant at one scale (i.e. migration over thousands of kilometres) is correlated with a cognitive difference detectable at another scale (i.e. feeders separated by a few metres). For example, birds that store food at thousands of locations over vast home ranges outperform related species that do not rely on stored food for survival, on both room-sized and operant chamber spatial memory tests (Brodbeck & Shettleworth 1995). Although a comparison between the memory abilities of a single pair of migrant and nonmigrant subspecies does not constitute a critical test of the hypothesis that the evolution of migration involves enhanced spatial memory, it is an important first step. Other pairs of migrant and nonmigrant populations should be compared to test the generality of the association between enhanced spatial memory and migration behaviour. If the relationship holds across numerous evolutionarily independent comparisons, this would suggest that natural selection for accurate migration has led to changes in cognitive abilities that transcend spatial scales.

Because of the well-established link between the hippocampus and spatial memory in food-storing birds, we examined the relative investment in the hippocampus of migrant and nonmigrant juncos. We found that migrant juncos had more densely packed hippocampal neurons. This suggests that the hippocampus might be the neural substrate responsible, in part, for the enhanced spatial memory of migrant juncos. However, in contrast to the results from several comparisons between food-storing and non-food-storing birds and mammals (e.g. citations in Volman et al. 1997; Lavenex et al. 2000), we found no evidence that migrants have greater hippocampal volume relative to the rest of the forebrain. However, our conclusion is tentative because of low statistical power. There is some evidence of seasonal neurogenesis in the hippocampus of food-storing birds (Barnea & Nottebohm 1994; Smulders et al. 1995), but little else is known about the mechanism responsible for hippocampal enlargement in these species. In fact, it is not well understood whether the hippocampal enlargement of food-storing birds occurs within the lifetime of each individual or has occurred over evolutionary time (Clayton & Krebs 1994b; but see Cristol 1996). The greater relative volume of the hippocampus reported for many food-storing birds and mammals could arise through several mechanisms, such as producing larger or more neurons or glial cells, sending more axonal projections from each neuron, possessing a relatively higher water content in the hippocampus (e.g. Yaskin 1984), or even shrinking of the rest of the telencephalon (Healy et al. 1996). Our results suggest the possibility that more hippocampal neurons could lead to enhanced spatial memory without greatly increasing the relative volume of the hippocampus.

If the relationship between migration behaviour and spatial memory, for which we found evidence in the present study, turns out to be general, further research

will still be necessary to determine which aspect of migration relies on spatial memory. One idea is that migration requires enhanced spatial memory because of the need to become familiar with multiple home ranges annually, but we were unable to test this because statistical power was too low to allow reliable analyses. If there really is no difference in neuron density/size or hippocampal volume between nonmigrant individuals that remain on the same territory year-round and those that wander away from nesting territories in winter and establish more than one home range annually, this would suggest that some facet of the migratory journey is responsible for the differences in hippocampal neurons between migrants and nonmigrants. Alternatively, differences in some aspect of the breeding range of the two subspecies may affect hippocampus volume. Although there has been some suggestion that habitat complexity can have detectable effects on avian hippocampal volume (Abbott et al. 1999), we are aware of no systematic differences in nesting habitat structure between the two subspecies that would predict hippocampal or spatial memory differences. Migration seems a more likely candidate. Lesion studies on homing pigeons and migratory sparrows have demonstrated that the hippocampus, although not necessary for orientation per se, does enhance homing through its role in landmark learning and recognition (Strasser et al. 1998). Even if much of a migratory flight is carried out high above a darkened landscape using a genetic programme, accurately relocating home ranges and critical stop-over sites may require enhanced spatial memory and more hippocampal neurons. Thus, while differences in the spatial memory and hippocampus of migrant and nonmigrant juncos were not as dramatic as those between some food-storing and non-food-storing birds, the tentative conclusion from our study is that the evolution of migration in dark-eyed juncos has led to an improved spatial memory and increased hippocampal neuron density.

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