Annual Cycle of the Black-Capped Chickadee: Seasonality of Food-Storing and the Hippocampus

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Key Words
Avian hippocampus • Seasonal neuroplasticity • Food caching • Food hoarding • Adult neurogenesis • Poecile atricapillus • Poecile atricapilla • Parus atricapillus

Abstract
Previous research presents a mixed picture of seasonal variation in the hippocampus of food-storing black-capped chickadees. One field study has shown an October peak in hippocampus volume, although laboratory studies conducted to determine whether photoperiod regulates this seasonal growth have failed to find changes in the size of the hippocampus. To resolve the discrepancy between field and lab reports we examined caching activity, hippocampal volume, and neurogenesis in adult male black-capped chickadees at four times over the annual cycle: October, January, April and July. We found that more birds stored food in October than at other times of year, but did not observe a significant change in the size of the hippocampus over the annual cycle. Telencephalon volume, however, was larger in October than in July. Hippocampal neuronal recruitment showed a significant peak in January, but there was no seasonal change in neuronal recruitment in the adjacent hyperpallium apicale. These results indicate that there might be seasonal variation in the recruitment of new neurons into the hippocampus of chickadees without overall seasonal change in hippocampal size.

Introduction
Seasonal neuroplasticity occurs in a variety of bird species. The best known example of seasonal brain change is in the vocal control system of songbirds. In spring, when birds sing the most, many song control nuclei dramatically increase in size. The seasonal growth of song nuclei such as HVC, robust nucleus of the arcopallium (RA) and area X can be attributed to a number of physiological changes such as increased cell spacing, increased soma size and incorporation of adult-generated neurons [for review see Tramontin and Brenowitz, 2000]. The primary proximate factor stimulating seasonal growth of the song control system is photoperiod. In the laboratory, switching birds from short, winter-like days to long, spring-like days results in rapid growth of the song system via gonadal recrudescence and a surge in testosterone.

The hippocampus is another neural structure that shows seasonal change. Food-storing black-capped chickadees, Poecile atricapillus, cache thousands of individual food items over large areas in fall and winter. Items of food are later retrieved using hippocampus-dependent spatial memory [for review see Shettleworth, 2003]. In a seasonal field study examining hippocampal volume in adult and juvenile chickadees of both sexes, Smulders et al. [1995] found a peak in hippocampus volume in October, the month when most storing was assumed to occur [Odum, 1942; Smulders et al., 1995; but see Pravosudov, 2006].
A number of investigations have since examined whether seasonal growth of the chickadee hippocampus is initiated by changing day length as in the song control system. Specifically, investigators have sought to determine whether decreasing day-length, as would be experienced in the fall, triggers hippocampal enlargement. The first study compared caching rates and hippocampal volume in two groups of chickadees both caught in the wild in spring [Krebs et al., 1995]. One group of birds was continuously housed on short-days (short-day group) whereas the other group was first housed on long-days and then switched to short-days after two months (long-day group). Although the birds in the long-day group significantly increased caching activity when switched to short days compared to birds held on constant short days, the hippocampus did not show a corresponding enlargement. Similar results were found in a study conducted by MacDougall-Shackleton et al. [2003]. In their study, switching birds from short to long days in the lab significantly decreased caching activity compared to birds held on constant short days but no differences in hippocampal volume were observed between birds housed under the different photoperiodic conditions. In another study examining photoperiodic control of neurogenesis in the chickadee hippocampus, no change in hippocampal volume was detected when birds were switched from long to short days [Hoshooley et al., 2005]. It is perhaps surprising that changing day-length does not affect the size of the hippocampus given that photoperiod is such an important factor regulating neuroplasticity in the song-control system. Caching experience itself might be a more important factor in this regard. Developmental studies show that storing and retrieving experience initiates hippocampal growth in juvenile mountain chickadees [Clayton, 2001]. Although differences in food-storing were observed in two of the experiments described above, the reduced opportunities for food-storing in captivity could have prevented substantial hippocampal growth.

Given the importance of possible seasonal change in such a large neural structure as the hippocampus, we examined the phenomenon in a wild population of birds. We collected adult, male black-capped chickadees from the wild at four evenly spaced times of year (October, January, April and July) and measured caching activity, hippocampal volume, and neurogenesis in these birds. Caching activity was assessed by observing birds in an aviary for four days. Neurogenesis was examined by administering the cell birth marker 5-bromo-2’-deoxyuridine (BrdU) and visualizing labeled cells immunocytochemically. The data collected in this study provide a profile of annual changes in hippocampal morphology in a wild population of adult black-capped chickadees.

Methods

Subjects
Twenty-four adult male black-capped chickadees were captured at four times of year: 15 October to 15 November 2002 (n = 6), 15 January to 15 February 2003 (n = 5), 15 April to 15 May 2003 (n = 6), 15 July to 15 August (2002: n = 3; 2003: n = 4). Birds were captured from a banded population approximately 30 km from the University of Western Ontario, London, Canada (43.02° N, 81.15° W). Sex was determined in the field by body size and later confirmed by examination of the gonads during dissection. Age was determined through previous banding records and only adult (minimum 10 months old) birds were used in this study. Birds were housed in cages in a rooftop enclosure to maintain exposure to natural photoperiod and weather conditions. Cages contained a wooden nest box, several natural coniferous branches, and wooden perches. Birds were given free access to water and food (ground sunflower seeds and Mazuri small bird maintenance diet supplemented with whole sunflower seed), except prior to food-storing sessions (see below). The birds used here were also the subjects of a parallel study examining seasonal changes in the song control system [Philmore et al., 2006]. All experimental procedures were conducted under a University of Western Ontario animal care protocol according to the guidelines of the Canadian Council of Animal Care.

BrdU Administration and Behavioral Assessment
On the day following capture all birds received four injections of BrdU (Sigma, St. Louis, MO) delivered 2 h apart. All injections were administered to the pectoral muscle at a dose of 75 μg/g in 0.1 M phosphate-buffered saline (PBS; pH = 7.4; 0.005 ml/g body weight of BrdU solution prepared as 0.015 g/ml). In July 2002 when this study began, the three birds we caught were administered an additional, fifth injection of BrdU, delivered 2 h prior to perfusion on day seven of this experiment. Because we decided to administer only the first four injections of BrdU to all other birds caught for this study, we did not examine neurogenesis in the three birds captured in July 2002.

On day three in captivity, the assessment of food-storing behavior and general activity began. Birds were observed individually in an indoor aviary containing several large tree branches in which holes had been drilled to facilitate food-storing activity. Behavior was recorded on a computer by an observer viewing the aviary through one-way glass. Recorded behaviors included: eating, drinking, storing, retrieving and preening. Cache site locations and the various behaviors were recorded using assigned keys on the computer keyboard. General activity levels were assessed by recording all movements from one perch location to another during a session. All food-storing sessions occurred within the first few hours following sunrise. Each morning birds were taken from their home cages on the roof to the indoor aviary where the storing observations occurred and after these sessions the birds were returned to the roof. On the day preceding a storing session, food was removed from the birds’ home cage at dusk. On day three...
in captivity, birds were released into the aviary for a 20 min habituation session. On day four, birds were released into the aviary for 20 min; sunflower seeds were made available for storing after an initial 5 min habituation period. On days five, six, and seven birds were observed in the aviary first for 10 min with no access to food (to score retrieval of previously stored food items) and then for 15 min with access to sunflower seeds (to score storing activity). To compare general locomotor activity over the year we tallied the total number of movements from one perch location to another that occurred during the second 5 min of the observation sessions on days five, six, and seven (during the last half of the initial retrieval phase).

**Perfusion and Histology**

Birds were perfused after the food-storing session on day seven. Birds were killed by an overdose of ketamine and xylazine and transcardially perfused with heparinized PBS (pH 7.5) followed by 4% paraformaldehyde. The brain was removed from the skull, weighed to the nearest 0.1 mg, submerged in 4% paraformaldehyde in PBS for 24 h, weighed again, and then cryoprotected in 30% sucrose in PBS until saturated (approximately 24 h). The brains were then weighed a third time, frozen on pulverized dry ice, and stored at −70 °C until further processing.

Brains were sectioned on a cryostat in the coronal plane at 30 µm thickness. Two series of sections were collected for this study: one for Nissl staining with thionin and one for immunochemical staining of BrdU. Every third section was collected for Nissl staining (90 µm intervals) and every twelfth section was kept for BrdU labeling (360 µm intervals). For the Nissl set, sections were collected in PBS, mounted on gelatin-coated slides, stained with thionin, serially dehydrated and coverslipped using Permount (Fisher Scientific).

For the BrdU set, sections were initially collected in cryoprotectant solution (30% sucrose and 30% ethylene glycol in buffer) and stored at −20°C. For processing, sections were transferred from the cryoprotectant into PBS and then washed twice more in PBS. Staining for BrdU with DAB immunocytochemistry using mouse anti-BrdU, 1:35 (BD Biosciences, Cat. No. 347580) was carried out using the protocol described in Hoshooley and Sherry [2004]. Brains were processed in two batches of twelve brains in March, 2005. Each batch contained brains from all times of year examined in this study.

**Morphometry and Cell Counting**

In Nissl-stained sections, the volume of the hippocampus was calculated by measuring the region’s area, according to the boundaries described by Sherry et al. [1989] in every third mounted section (270 µm intervals); area estimates were then combined using the formula for the volume of a truncated cone [Sherry et al., 1989]. Using the same method, the volume of the telencephalon was estimated by measuring the structure in every second mounted section (180 µm intervals). Area measurements were made using Spot Imaging software from digital images captured with a Spot Insight camera mounted on a Zeiss Axioskop microscope using a 5× objective for the hippocampus and a 1.25× objective for the telencephalon. All measurements were made by a researcher blind with respect to the birds’ group.

To estimate the number of new cells recruited into the hippocampus (one week following BrdU administration), we examined all sections labeled for BrdU and counted all labeled nuclei appearing in that brain region according to the morphological criteria of Gould et al. [1999] namely: darkly stained, rounded oval nuclei approximately 10 µm in diameter. Using these criteria, it is possible that some non-neuronal cells might be included in our counts, most likely large glial nuclei. However, in a recent study [Hoshooley and Sherry, pers. obs.], we found a highly significant positive correlation between counts of Hp DAB-stained tissue and counts obtained using double labeling for BrdU and the neuron specific marker NeuN (Neuronal Nuclei specific protein). In the sections examined (every 12th section, 360 µm apart), labeled cells were counted exhaustively in both hemispheres. This method of counting is appropriate for estimating neuronal recruitment and has been used previously for cell counts in the chickadee hippocampus [Hoshooley and Sherry, 2004; Hoshooley et al., 2005]. To estimate the density of BrdU-labeling in the hippocampus (number of neurons per mm³), we divided the average number of labeled neurons in a section by the average volume of the section. We calculated the volume of an Hp section by multiplying the area of the section by the section thickness, giving the volume in which the new neurons were counted. To estimate the total number of newly recruited hippocampal neurons we multiplied the density of BrdU labeling by hippocampus volume. For each new neuron that was counted, the distance traveled from the subventricular zone directly beneath the hippocampus was measured. New hippocampal neurons originate from neural progenitor cells residing in the subventricular zone (SVZ), the cell layers lining the lateral ventricles. For each new neuron recorded in this study, we measured the distance traveled from the SVZ by tracing with a cursor on the digitized image of the section the most direct perpendicular straight line path from the center of the new neuron to the center of the SVZ. Although we do not know for certain whether new neurons travel along a straight, perpendicular trajectory from the SVZ into the hippocampus, this seemed to be the most direct way to assess displacement from the SVZ during neuronal migration and is similar to methods used by Barnea and Nottebohm [1994]. We also compared hippocampal neuron recruitment along the rostrocaudal extent of the structure within three distinct subdivisions delineated by Barnea and Nottebohm [1994].

Mitotic activity in the subventricular zone directly adjacent to the hippocampus was also assessed in this study. The same criteria used for the hippocampus were used to record the number of BrdU-labeled cells in the SVZ except that no size criterion was applied to labeled cells. The number of BrdU-labeled cells in SVZ (adjacent to the hippocampus) of both hemispheres was recorded for all sections in which the hippocampus appeared. The total number of BrdU-labeled cells recorded was divided by the number of sections counted in order to obtain an average number of BrdU-labeled Hp SVZ cells per section. We also assessed BrdU-labeling in the hyperpallium apicale [HA, according to the boundaries described by Hoshooley and Sherry, 2004] and in the SVZ directly adjacent to HA using the same procedure as for the hippocampal SVZ.

**Statistical Analyses**

All dependent variables, unless otherwise stated, were analyzed using one-way analysis of variance (ANOVA) with time of year as the between subjects factor (October, January, April, and July). All analyses with p < 0.05 were considered statistically significant. Significant ANOVAs were further analyzed using Tukey’s HSD post-hoc tests.
Results

Food-storing occurred at relatively low rates compared to previous studies of captive birds, possibly because the birds were transported daily between the outdoor aviaries and the indoor storing arena. Half of the birds (3/6) collected in October exhibited food-storing, whereas only two birds collected at other times of year stored any seeds (2/18; \( \chi^2 = 4.13, \text{d.f.} = 1, p < 0.05 \)). Expected frequencies for this \( \chi^2 \) test are low for some cells, but satisfy the conditions of Koehler and Larntz [1980] for \( \chi^2 \) tests with some low expected cell frequencies. We observed no significant differences in general locomotor activity (number of perch changes) across the annual cycle (\( \text{F}(3,20) = 1.99, p = 0.34 \)).

We did find, however, significant seasonal fluctuation in telencephalon size (\( \text{F}(3,20) = 3.144, p = 0.048 \); fig. 1), as previously reported [Phillmore et al., 2006]. Post-hoc analyses revealed the telencephalon was significantly larger in October than in July (\( p < 0.05 \)). Approximately 73% of all new neurons recorded for the hippocampus (n = 81) were found within 49 \( \mu \text{m} \) of the subventricular zone as shown in figure 3. A repeated measures ANOVA with Hp region (rostral, middle, caudal) as a within subjects factor showed no significant differences in recruitment along the rostrocaudal axis of the hippocampus (\( \text{F}(2,40) = 1.342, p = 0.273 \)). We also examined mitotic activity in the subventricular zone directly ventral to the hippocampus but found no significant fluctuations in this measure across the annual cycle (\( \text{F}(3,20) = 1.411, p = 0.274 \); fig. 4).

Finally, we examined neuron recruitment in the hyper pallium apicale (HA), a structure adjacent to the hippocampus. We found no significant differences in recruitment to HA across the times of year examined (\( \text{F}(3,20) = 0.733, p = 0.546 \); fig. 4), nor did we find any significant differences in mitotic activity in the subventricular zone beneath HA (\( \text{F}(3,20) = 0.916, p = 0.454 \); fig. 4) over the seasons examined.

Discussion

In this study more chickadees stored food in October than at the other times of year. This finding provides some support for the notion that there might be a peak in seasonal changes in overall hippocampus size, both with and without correction for annual variation in brain mass and telencephalon size.

In the present study we observed significant seasonal fluctuation in both measures of hippocampal neuron recruitment (density of Hp BrdU-labeling, \( \text{F}(3,17) = 5.511, p = 0.008 \) and total number of Hp BrdU-labeled neurons, \( \text{F}(3,17) = 5.135, p = 0.010 \), see fig. 2). Post-hoc analyses of both measures revealed significantly greater hippocampal neuron recruitment in January than in October (\( p < 0.05 \)) or in July (\( p < 0.05 \)). Approximately 73% of all new neurons recorded for the hippocampus (n = 81) were found within 49 \( \mu \text{m} \) of the subventricular zone as shown in figure 3. A repeated measures ANOVA with Hp region (rostral, middle, caudal) as a within subjects factor showed no significant differences in recruitment along the rostrocaudal axis of the hippocampus (\( \text{F}(2,40) = 1.342, p = 0.273 \)). We also examined mitotic activity in the subventricular zone directly ventral to the hippocampus but found no significant fluctuations in this measure across the annual cycle (\( \text{F}(3,20) = 1.411, p = 0.274 \); fig. 4).
food-storing activity in autumn, with most storing occurring sometime between August and December. Recently Brodin [2005] observed food-storing in a free-living population of black-capped chickadees and found peak storing in September that slowly declined through the autumn. Although Brodin observed a higher latitude population than ours, we presume autumn is when storage by black-capped chickadees peaks over most of their range.

Despite changes in food storing and despite having sample sizes comparable to previous studies, we did not find significant seasonal change in the hippocampus in contrast to the previously reported October increase in hippocampal size [Smulders et al., 1995]. It is possible, as suggested by Krebs et al. [1995], that seasonal change in the hippocampus could differ in different populations, and this might account for the discrepancy between the present results and those of Smulders et al. [1995]. It is also possible that differing environmental conditions during the years in which these two studies were conducted could explain the different results obtained. Differences in the length of time that birds were held in captivity could also potentially account for the differing results. Captivity is known to depress hippocampal neuron recruitment [Barnea and Nottebohm, 1994; Hoshouley and Sherry, 2004] and it is possible that the volume of the hippocampus might also be affected by captivity as is the case in dark-eyed juncos [Smulders et al., 2000].

If hippocampal volume does fluctuate over the annual cycle in the chickadee, seasonal growth is clearly not as consistent or as substantial as the seasonal growth documented for the song control system [Tramontin and Brenowitz, 2000]. Similar to our study, an examination of hippocampal neuron recruitment in wild caught chickadees from October to March did not detect any significant change in the size of the hippocampus [Hoshouley and Sherry, 2004]. Also consistent with our results are photoperiodic manipulations that have successfully altered caching activity in chickadees, but have not led to corresponding changes in hippocampal size [Krebs et al., 1995; MacDougall-Shackleton et al., 2003; Hoshouley et al., 2005]. Although more chickadees in this study were found to store food in the fall, instances of caching were observed during three of the four times of year we examined, suggesting that food-storing might occur at some level all year. If this is so, then one might not expect dramatic seasonal changes in the neural structure associated with this behavior. Large seasonal volume changes found in the song control system are concomitant with obvious and discrete changes in singing behavior, including changes in singing rate and song stereotypy [e.g., Smith et al., 1997]. Furthermore, the song control system appears dedicated to the control of song and perhaps other learned vocalizations. The hippocampus, although it shows volumetric specialization correlated with food-storing behavior, surely continues to serve other non-food-storing functions in food-storing birds, functions which presumably are necessary throughout the year [Colombo and Broadbent, 2000]. If this is so, then one might not expect to observe large fluctuations in hippocampal size across the annual cycle.

Seasonal stability of hippocampal size in black-capped chickadees is consistent with results with adult eastern gray squirrels. Lavenex et al. [2000] studied squirrels captured in October, January, and June, but found no differences in hippocampal volume. Only one other study of the avian hippocampus has revealed seasonal fluctuation in structure size. Two species of South American brood-parasitic cowbirds showed enlargement of the hippocampus during the spring breeding season [Clayton et al., 1997]. In screaming cowbirds, Molothrus rufoaxillaris, males and females search for host nests together and both males and females of this species showed increased hippocampal size in the spring breeding season. Among shiny cowbirds, M. bonariensis, only females engage in nest searching and only females showed hippocampal enlargement in the spring. However, Clayton et al. [1997] caution that the observed seasonal changes might be confounded by the age distributions of their samples at the times of year they were collected and that their results should be replicated in birds of known ages.

As previously reported by Phillmore et al. [2006] we found that the telencephalon was larger in October than in July. We also found that post-perfusion brains from July were heavier than brains from any other time of year. Furthermore, July brains showed the greatest shrinkage following cryoprotection. These data replicate earlier reports of seasonal changes in overall brain size and osmolality [Smulders et al., 1995; Smulders, 2002]. Increases in brain mass probably result from increases in the water content of the brain, although the factors regulating water content are largely unknown [Smulders, 2002]. Collectively, these results demonstrate that caution is necessary when interpreting seasonal changes in absolute brain measures. In the current study, it is unlikely that the seasonal changes we detected in telencephalon volume, brain mass, and brain shrinkage affected our results regarding the hippocampus because we took both absolute and relative measures of volume that should correct...
for any difference in tissue shrinkage following cryo-protection.

In the present study we found a January peak in the recruitment of new neurons to the hippocampus (as assessed one week following BrdU administration), but did not find seasonal differences in mitotic activity in the subventricular zone directly beneath the hippocampus. Seasonally changing neuron recruitment did not, however, extend to the adjacent hyperpallium apicale. These results differ somewhat from those first reported for seasonal neurogenesis in the chickadee hippocampus. Barnea and Nottebohm [1994] showed hippocampal recruitment of new neurons to be greatest in October, but it is difficult to directly compare their results to ours because they examined 6-week survival of new neurons whereas we only examined 1-week survival. A more comparable study that examined recruitment of new neurons between 5 and 20 days following administration of BrdU in the period from October to March found no significant seasonal change, although there was some indication of heightened recruitment in January [Hoshooley and Sherry, 2004]. By examining only adult male chickadees in the current study we eliminated two potential sources of variability in the previous study, age and sex, and thus should have been better able to detect a significant January peak in hippocampal neuron recruitment if present. In the current study, we observed an overall lower level of hippocampal neuron recruitment than in the previous study [Hoshooley and Sherry, 2004]. Our exclusion of juveniles and females from the current study might have contributed to the lower level of recruitment we observed. It is also possible that environmental or population differences between the years and locations in which the two studies were conducted could explain the differing levels of recruitment that were observed. The function of new neurons that are recruited into the hippocampus remains unknown. Perhaps more new neurons are required in January to ensure efficient encoding of new cache locations following intense caching in autumn and heavy processing demands on the hippocampus. Some models and experimental results indicate that hippocampal neurogenesis in mammals can disrupt memories encoded before the period of neurogenesis while improving retention of memories acquired afterward [Meltzer et al., 2005]. Disrupting old and potentially interfering memories could be a benefit of neurogenesis, quite apart from any advantage it confers in the storage of new memories. It has been proposed that new neurons are required to break existing network connections and clear old memories traces in the mammalian brain [Feng et al., 2001].

Because we found no significant changes in the volume of the hippocampus, the increased neuron recruitment we observed is likely to be part of a process of neuronal replacement, as first proposed by Barnea and Nottebohm [1994]. Although the picture of seasonal neuronal recruitment in the chickadee hippocampus is not completely clear, it does appear that neuron recruitment is heightened during the part of the annual cycle when chickadees are engaged in storing and retrieving food. The results presented here, in conjunction with other studies, indicate that seasonal changes in hippocampus size are not regularly observed phenomena in black-capped chickadees. Seasonal change in hippocampal neurogenesis is more routinely observed although the seasonal timing varies among studies.

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References


