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Variation in memory and the hippocampus across populations from different climates: a common garden approach

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Selection for enhanced cognitive traits is hypothesized to produce enhancements to brain structures that support those traits. Although numerous studies suggest that this pattern is robust, there are several mechanisms that may produce this association. First, cognitive traits and their neural underpinnings may be fixed as a result of differential selection on cognitive function within specific environments. Second, these relationships may be the product of the selection for plasticity, where differences are produced owing to an individual's experiences in the environment. Alternatively, the relationship may be a complex function of experience, genetics and/or epigenetic effects. Using a well-studied model species (black-capped chickadee, *Poecile atricapillus*), we have for the first time, to our knowledge, addressed these hypotheses. We found that differences in hippocampal (Hp) neuron number, neurogenesis and spatial memory previously observed in wild chickadees persisted in hand-raised birds from the same populations, even when birds were raised in an identical environment. These findings reject the hypothesis that variation in these traits is owing solely to differences in memory-based experiences in different environments. Moreover, neuron number and neurogenesis were strikingly similar between captive-raised and wild birds from the same populations, further supporting the genetic hypothesis. Hp volume, however, did not differ between the captive-raised populations, yet was very different in their wild counterparts, supporting the experience hypothesis. Our results indicate that the production of some Hp factors may be inherited and largely independent of environmental experiences in adult life, regardless of their magnitude, in animals under high selection pressure for memory, while traits such as volume may be more plastic and modified by the environment.

Keywords: captivity; cognition; natural selection; neurogenesis; plasticity; spatial memory

1. INTRODUCTION

Enhancements to cognitive traits are generally associated with enhanced neural structures that support those traits [1]. For example, many species with high demands for spatial processing tend to possess enhanced features in the hippocampus (Hp), the area of the brain partially responsible for spatial memory [2]. This pattern may be observed within as well as across species. For example, spatial learning and memory use can produce changes in the Hp, including gene expression in rodents [3] and structural and physiological changes in rodents and humans [4–8].

One potential factor that may produce natural variation in memory dependency, and consequently, variation in the Hp of some species, is climatic or environmental severity. For example, animals that cache and retrieve food at a later time may experience more pressure for memory accuracy in harsh climates. Our previous multi-year work on multiple populations of food-caching black-capped chickadees (*Poecile atricapillus*) demonstrated a strong and robust positive relationship between a population's winter climatic severity, spatial memory and Hp attributes [9–12]. Populations along a gradient of climatic severity in North America showed differences in Hp features of up to 40 per cent [9–12]. However, as of yet, we have been unable to separate the possible mechanisms responsible for the production of enhanced spatial memory and its associated neural structures in birds experiencing more harsh environments.

One possible explanation for the positive relationship between enhanced spatial memory and the brain is that natural selection may have produced fixed, specialized cognitive and Hp traits that enhance survival for individuals living in harsh environments with high cognitive demands [2,13]. In the case of food-caching birds that rely on spatial memory to recover food, in environments where alternative food resources are low and energetic demands are high, selection for advanced spatial memory and enhanced Hp features should be high [2]. Assuming there is a cost to maintaining advanced neural features [14], selection should not favour the same neural advancements in more mild areas where they may not be necessary. This adaptive specialization hypothesis [13] then predicts more enhanced Hp features in more severe environments relative to more mild conditions. Likewise, these traits should be fixed and present before the animal ever experiences the need for cognitive demands, i.e. the selection pressure itself.

Although comparative data generally support the adaptive specialization hypothesis, an alternative explanation is that the differences in Hp morphology among populations are a result of differential memory-based food-caching and

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retrieval experiences imposed by large differences in environmental harshness (*sensu* [15,16]). In this case, selection may have resulted in the ability of the brain to be plastic, thus Hp attributes may be dependent upon cognitive experiences of the individual. In our food-caching example, animals that live in more severe (i.e. cold) environments tend to have higher metabolic costs, cache more food, probably use their memory more frequently to recover that food [9] and thereby may enhance their Hp architecture via increased memory use. Indeed, many previous studies suggest that the physical environment and an individual's memory use may play an important role in altering brain structure and processes such as neurogenesis (e.g. [4–7]). For example, comparisons of captive and wild animals suggest that the reduced cognitive stimulation in a captive environment may lead to reduced Hp volume and neurogenesis [17–19]. Similarly, memory experience itself has been suggested to have an effect on neurogenesis levels [5,19]. Thus, differences in the formation and maintenance of cognitive and Hp traits may simply be a function of an individual's experiences.

In reality, however, the creation of complex neurobehavioural traits is probably not a simple, mutually exclusive relationship between genetics and the environment, but a more complex phenomenon. Indeed, most neural features and patterns are produced as some combination of genetic and environmental expression [20]. For example, the expression of schizophrenia may require both a genetic component of reduced neurogenesis as well as an environmental effect on the central nervous system during development [21]. Even within the brain, not all traits may respond in the same way, as some neural features are known to be quite plastic (e.g. dendrites and dendritic spines; see Roth *et al.* [22] and references therein).

Using a well-established food-caching bird model, we have taken a first step in addressing the mechanisms underlying the association between harsh environments and enhanced neural structures supporting spatial memory. We controlled the environment experienced by birds during the bulk of development and the hypothetically important caching and retrieving period (autumn/winter), thereby eliminating differences in climate and the natural levels of caching, memory use and movement as explanations for the observed relationship. We recognize that we have not controlled for more complex gene–environment interactions, but our goal is to take a first step in unravelling this complex relationship. As we have controlled the major environmental features in which the animals live, any difference observed between the two populations in captivity could be attributed in large part to inheritance (either genetic or maternal effects). If, however, no differences are observed in the behaviour and morphology in these captive-raised animals, we could conclude that the environmental differences experienced in the wild are indeed largely important for the variation previously observed. In either case, though, this is not an all-or-nothing determination. We examined individuals from our model species from the two populations that experience the largest differences in winter conditions and possess the largest differences in Hp attributes (Alaska and Kansas) [10–12]. Given the severe conditions in Alaska, birds from this population cache more food, perform more accurately in spatial memory tasks and possess increased Hp attributes relative to those from the south [9].

2. METHODS

(a) *Study species*

Black-capped chickadees (*P. atricapillus*) were collected for the common garden (i.e. hand-rearing under identical conditions) aspect of this study during May and June 2009 from the latitudinal extremes of their range (Anchorage, Alaska: 61°10′ N, 149°53′ W; Manhattan, Kansas: 39°08′ N, 96°37′ W). We also compare the morphological data collected from these hand-raised birds with those obtained from wild-caught individuals collected from the same populations in the autumn of 2007 (Kansas; [10]) and 2008 (Anchorage; [12]) (see §2*e* below).

(b) *Hand-rearing and housing captive chickadees*

Twelve birds from each of the two populations were collected from different nests to avoid pseudoreplication. Chicks were collected at approximately 10 days of age and hand-raised indoors in laboratory conditions throughout the summer, autumn and winter seasons. Birds were collected when they were still in nesting cavities and had not yet opened their eyes. As we collected nestlings well before they left the nest and before they had any visual experiences, we prevented differences in caching-based memory experiences between the groups, although we did not exclude the impact of environmental factors that occurred prior to collection or maternal effects. To retain consistency in the hand-raising environment between our two field sites, the temperature, lighting and all techniques were similar from the first day of collection. When chicks were approximately 18 days old, they were transported to the University of Nevada, Reno, by plane from Alaska and by vehicle from Kansas. The same boxes were used for transport and the lighting and temperature conditions were similar in both cases. A detailed description of collection and housing procedures can be found in the study of Roth *et al.* [23].

All chicks were fed a diet of Orlux 'Handmix' formulae (Versele-Laga, Deinze, Belgium), wax worms (*Pyralidae* sp.), meal worms (*Tenebrio molitor*), phoenix worms (*Hermetia illucens*), crickets (*Acheta domesticus*), a slurry (consisting of dog food (Canidae, San Luis Obispo, CA, USA), cat food (Natura EVO, Santa Clara, CA, USA), Orlux insect patee premium and Orlux 'Handmix'), and nuts (pine nuts (*Pinus koraiensis*), peanuts (*Arachis hypogaea*) and sunflower seeds (*Helianthus* sp.)). Food types were systematically cycled throughout the day and were offered every 20 min during daylight hours. Food (less formulae) and water were provided ad libitum after birds reached independence (approx. 30–35 days after hatching).

During hand-rearing, chicks were housed in groups of four to six individuals in 17 × 17 × 24 cm wood boxes filled with sawdust to simulate nest cavities. At the fledgling stage (approx. 18–20 days after hatching), chicks were housed in pairs in 120 × 42 × 60 cm wire cages. At the dispersal stage (approx. 60 days after hatching), all birds were housed individually in their permanent arrangement in 60 × 42 × 60 cm wire cages in a 'Male/Female/Female/Male' arrangement. The populations were also systematically partitioned as 'Alaska/Kansas/Alaska/Kansas' within these rows. This series of steps and final arrangement allowed birds to retain sociality while preventing aggressive interactions.

The cage design of each bird was identical. In addition to food, water and a cuttlebone, each cage held one caching

block and one set of caching pockets (see §2*c* below for descriptions). These caching features were included in the cages to allow the birds to become familiar with caching in these structures (see also [9,19]). Birds could cache and retrieve in these pockets freely. There were no differences between the groups in the number of caches made in these pockets during the study (sum of 10 collections occurring every 2 days during the peak of caching in October/November 2009; $t_{22} = 1.056$, $p = 0.303$). These caching features were removed during food deprivation and replaced intact after food was returned (see below).

The two populations were held on the same light cycle. The summer cycle was a 15 L : 9 D cycle beginning from the day of collection for both populations. Beginning in early August until mid-October, the light cycle gradually shifted (approx. 0.5 h week^{-1}) to a winter cycle of 9 L : 15 D. All birds were maintained on the winter light cycle for the reminder of the study.

(c) *Spatial memory comparison between hand-raised populations*

In late autumn and throughout winter 2009, we performed behavioural tests to determine if there were differences in spatial memory capabilities and caching propensities between the two groups.

(i) *Testing room*

The propensity to cache and a cache/retrieval experiment were conducted in the testing room. The testing room was adjacent to the rooms where the birds were housed. Access from the bird's home cage to the testing room was through an opening in the wall connecting each individual bird's cage with the testing room, and bird movement was prompted with light manipulation (e.g. [24]).

The testing room (218 × 373 × 263 cm) consisted of two perching trees (cut aspen) in the centre of the room with caching locations on the walls. The caching locations comprised both boards (18 × 31 cm) with 10 rubber pockets (2.5 × 4.0 cm) and blocks (9.0 × 14.5 × 4.0 cm) with a single hole drilled into them [9], identical to the pockets and blocks available in their home cages. All boards and caching blocks contained perches. Each caching pocket had a flap covering the opening, so that subjects had to lift the flap to recover food from the pocket. Likewise, each block's caching hole could be covered with a string with a knot tied in the end. The knot was suspended above the hole so the subjects had to remove the knot from the hole to acquire food items. We hung 11 boards and 10 blocks on each of the two walls. Blocks were staggered between the caching boards. The opposite walls were identical in arrangement. Thus, there were 240 possible caching locations in the room. Prior to testing, each bird was allowed to habituate to the testing room for approximately 1 h d^{-1}, every third day, for a total of no less than 6 h [9].

(ii) *Cache/retrieval task*

The cache/retrieval experiment occurred in the morning hours of December 2009 approximately 1 h after lights were on. Birds were deprived of food for 1 h the previous night as well as during the experiment. During the caching and retrieving task, a dish with pine nuts and wax worms was provided in the testing room. In the testing room, we recorded the type and amount of food consumed, as well as the location of any caches made in the array. All observations of testing occurred from behind one-way glass. After 20 min, the bird was returned to its home cage and all caches were removed from the caching array. After a 6 h retention interval, we replaced the bird's caches in the appropriate caching positions, covered all caching sites and allowed the bird back into the testing room for 20 min. The only food available during this part of the task was located in the bird's previous cache locations; no food was available in the dishes. We recorded the total number of caches made, the number of caches recovered and the number and order of caching locations investigated. We considered the bird to have investigated a caching site if the bird lifted the flap of the pocket or removed the knot from the hole in the block [9,16]. We tested each bird five times, using the total amount of items cached and the average number of looks to retrieve the first food item for each bird in the analyses. After the test was completed, the birds were given ad libitum food and we allowed at least 2 days before retesting. If the bird did not inspect any location or if it did not find any caches during the five trials, it was dropped from the analyses.

(iii) *Associative learning spatial task*

An associative learning task was performed in the birds' home cages during January 2010 shortly after lights were on with birds in the cage row (i.e. two Alaska and two Kansas) tested simultaneously. We used a caching array of 1.5 cm wells drilled into a wooden board (size 40 × 18 cm; see also [23]) in a 3 × 5 grid arrangement. A black, craft 'pom-pom' ball was inserted into each well; all birds were able to remove the balls from the wells. After all birds were habituated to the boards (total duration of exposure >30 h), we placed one wax worm in one well of the testing board (chosen systematically for each bird), covered it and all other wells with the balls, and allowed the birds to recover the worm. For the next 5 days, the same board with all wells covered and a wax worm hidden in the same location was presented to each bird. We recorded the number of attempts to successfully locate the worm. We began our analysis with trial 2, as trial 1 is random by design. We ended our analysis with trial 6 as this is the point at which the score for both groups levelled. All trials were observed remotely with a live video feed to another room and recorded using Sony DCR-SR300 and DCR-SR47 digital video cameras on tripods. All birds performed the task (i.e. found the wax worm). One bird sustained an unrelated injury immediately prior to the trial and thus was tested after recovery.

(d) *Morphological comparison between hand-raised populations*

(i) *Brain preparation*

We compared the Hp volume, total number of Hp neurons and number of immature neurons of 12 birds from the two locations. All birds were sacrificed and their brains extracted on 2 February 2010 (approx. 8.5 months of age) following the work of Roth and co-workers [10,12]. Briefly, birds were anaesthetized (0.07 ml of 50 mg ml^{-1} Nembutal) and perfused transcardially with phosphate-buffered saline followed by 10 per cent methanol-free formalin (from paraformaldehyde). Brains were post-fixed for 7 days, cryoprotected and then frozen at −80°C for storage. Tissue was cut into 40 µm coronal sections on a Leica CM 3050S cryostat at −20°C. Tissue was divided into four series, with series one mounted directly and Nissl-stained, and series two processed for neurogenesis.

(ii) *Morphological measures*

Hp volume and neuron numbers were estimated every twelfth Nissl-stained section with modern stereological methods using StereoInvestigator software (Microbrightfield, Inc.) and a Leica microscope (M4000B). Both the Hp and telencephalon (Te) were measured in their entirety; Te was measured as a control for overall brain size. We measured the Hp as per Krebs *et al.* [2]. Brain volumes were estimated with the Cavalieri procedure [25]. Hp volume was measured with the optimal grid size of 200 μm, and the Te volume with a 1200 μm grid as determined previously [10,12]. Neuron counts were performed with an optical fractionator procedure [26] at 1000×. A 250 μm grid with a 30 × 30 μm counting frame, 5 μm dissector height and 2 μm guards was used as in previous studies of chickadees [10,12]. We calculated a coefficient of error (CE) to estimate precision with the nugget effect for both neuron counts (CE mean (s.e.) = 0.097 (0.005) and volume (CE mean (s.e.) = 0.015 (<0.001)) [27]. The left and right hemispheres were measured independently and summed to produce the reported total values.

(iii) *Doublecortin immunohistochemistry*

Immunohistochemistry was used to visualize the doublecortin protein (DCX), which is associated with neuronal cell microtubule machinery localized in newly developing neurons [28,29] in every twelfth tissue section. In passerine birds, DCX expression occurs for 25–30 days after the production of a new neuron, after which point expression of the protein ceases [28]. Consequently, the expression of this protein appears to be a relevant marker for young, developing neurons [30]. See LaDage *et al.* [19] for a full description of the benefits and limitations of the expression of DCX as a measure of neurogenesis.

The DCX-staining protocol was optimized for chickadees by LaDage *et al.* ([19]; see also [11]). Briefly, tissue sections were washed in Tris-buffered saline (TBS), and then incubated in 30 per cent hydrogen peroxide and TBS (1 : 50.0) for 30 min at room temperature. The sections were then washed in TBS and incubated at room temperature for 30 min in blocking buffer (normal horse serum (1 : 33.3), TX-100 (1 : 39.8) and TBS). The sections were then incubated overnight for approximately 18 h at 4°C in anti-doublecortin antibody (made in goats; 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA, SC-8066) and blocking buffer. On the second day, the tissue sections were washed in TBS and then incubated at room temperature for 2 h in biotinylated horse anti-goat antibody in blocking buffer (1 : 200, Vector Laboratories, Burlingame, CA, USA, BA-9500). The tissue sections were again washed in TBS and incubated at room temperature for 1 h in an ABC Elite kit (Vector Laboratories, PK-6100), followed by a DAB + nickel kit (Vector Laboratories, SK-4100) for 2 min 18 s at room temperature. The sections underwent a final series of washes in TBS and were mounted onto slides. The slides were dried at 37°C overnight and then lightly Nissl-stained.

Boundaries for the Hp were determined as per our previous studies [10,12,19]. The number of DCX-positive cells present in the Hp was estimated as above and followed our previous studies [11,19]. The counting frame was set at 70 × 70 μm on a 250 μm grid with a fixed dissector height of 5 μm at 1000× [11,19]. We calculated a CE to estimate precision with the nugget effect (CE mean (s.e.) = 0.111 (0.003)). The left and right hemispheres were measured independently and summed to produce the reported total values.

(iv) *Movement control*

To control for the possible effects of movement on our neurogenesis estimates, we video-taped and scored movement activity for all chickadees in October 2009 as per LaDage *et al.* [19]. The movement of each bird was monitored for 10 min and the number of hops taken and the total distance moved was recorded. There were no differences between the two groups in either hops ($t_{22} = 0.237$, $p = 0.815$) or distance moved ($t_{22} = 0.497$, $p = 0.624$).

(e) *Morphological comparison between hand-raised and wild-caught birds*

To ascertain if the morphological brain data we collected from hand-raised birds was comparable to data from wild-caught birds that did not experience captivity, we also compared the results of our common garden study with data published in our previous studies of wild individuals from the same Alaska and Kansas populations [10–12]. Wild-caught birds were collected in the autumn of 2007 (Kansas, $n = 13$) and the autumn of 2008 (Alaska, $n = 12$) using mist nets at feeders. The tissue was processed immediately upon capture following the same techniques as above in §2*d*. Likewise, histological analyses followed the same protocol in §2*d*. We note that because the two wild populations were sampled in different years (Kansas, 2007; Alaska, 2008; [10,12]), any variance owing to a potential year effect could not be ascertained.

(f) *Statistical analyses*

We analysed behavioural data with *t*-tests (cache/retrieval) and RMANOVA (associative learning). In both tasks, the number of looks to find the food was compared with random chance (cache/retrieval = 120.5; associative learning = 8). Although we include this analysis for the sake of completeness, the objective of the study was to compare the accuracy of spatial memory between the two groups. Thus, those are the main comparisons. As we expected Alaska to cache more and have better memory than birds from Kansas [9], these are directional tests.

We analysed all brain data with general linear models. We include analyses using Te volume (to control for brain size), Hp volume (to examine neuron density) and total neuron number (to control for proportion of DCX-labelled cells) as covariates. We also include analyses without covariates for completeness. We report least-square means (±s.e.) in these analyses. To control for inter-observer variation in analysis among different studies (see Roth *et al.* [22] for a discussion of this problem), all histological data reported here were collected exclusively by T.C.R. All data were measured blind to location. Data were log-transformed to meet the assumptions of normality and homogeneity of variance as necessary.

3. RESULTS

(a) *Spatial memory comparison between hand-raised populations*

(i) *Cache/retrieval task*

We first determined if there were differences in the amount of caches made by each group. Only birds that cached food in the testing room during the trial were included in this analysis. Chickadees from Alaska ($n = 8$) cached significantly more food items than those from Kansas ($n = 10$, $t_{16} = 2.193$, $p = 0.043$; figure 1*a*).

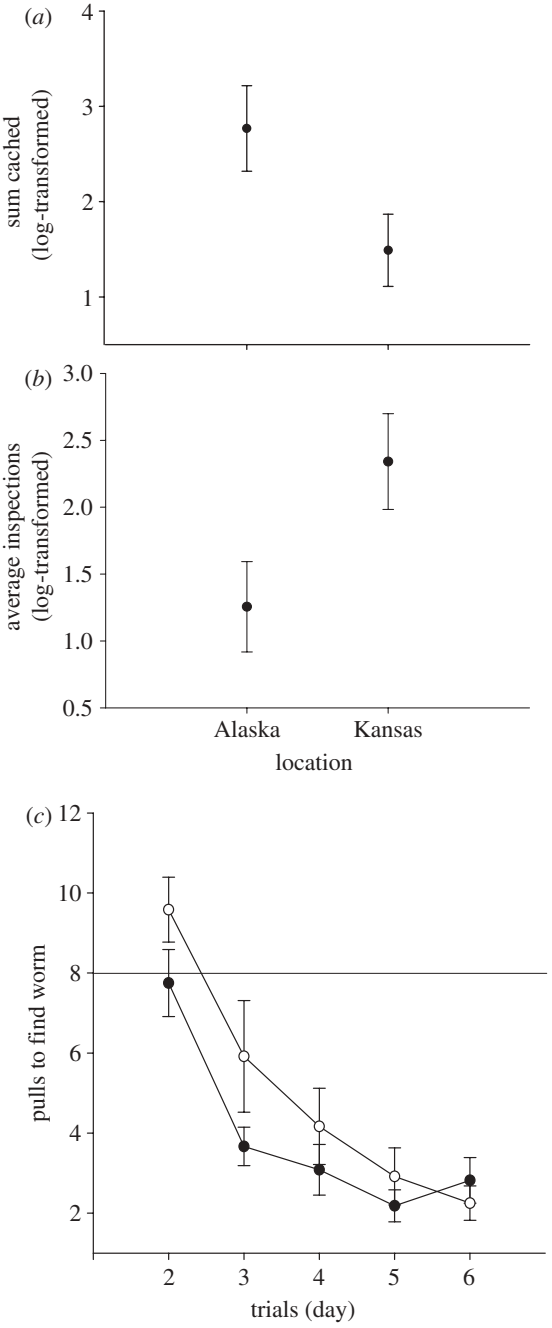

Figure 1. Hand-raised black-capped chickadees from populations with different demands for memory performed differently on memory tasks. Birds from Alaska (*a*) cached more food than those from Kansas in these trials. In addition, the Alaska birds (*b*) made fewer mistakes during a cache/retrieval task and (*c*) more quickly and more accurately learned an associative spatial task than those from Kansas. Both groups performed the tasks significantly more accurately than expected by random in both memory tests (all p's < 0.001); lines represent random chance performance; filled circles, Alaska; open circles, Kansas. Error bars represent $\pm$ 1 s.e.m.

We also determined if there were differences in the ability to retrieve those caches using spatial memory. Only the birds that retrieved their caches during the testing phase were included in this analysis. Alaskan chickadees ($n = 7$) inspected significantly fewer locations during the retrieval phase ($t_{13} = 2.186$, $p = 0.048$; figure 1*b*) than did the birds from Kansas ($n = 8$). Both groups retrieved their caches with significantly fewer inspections than expected by random (all p's < 0.001).

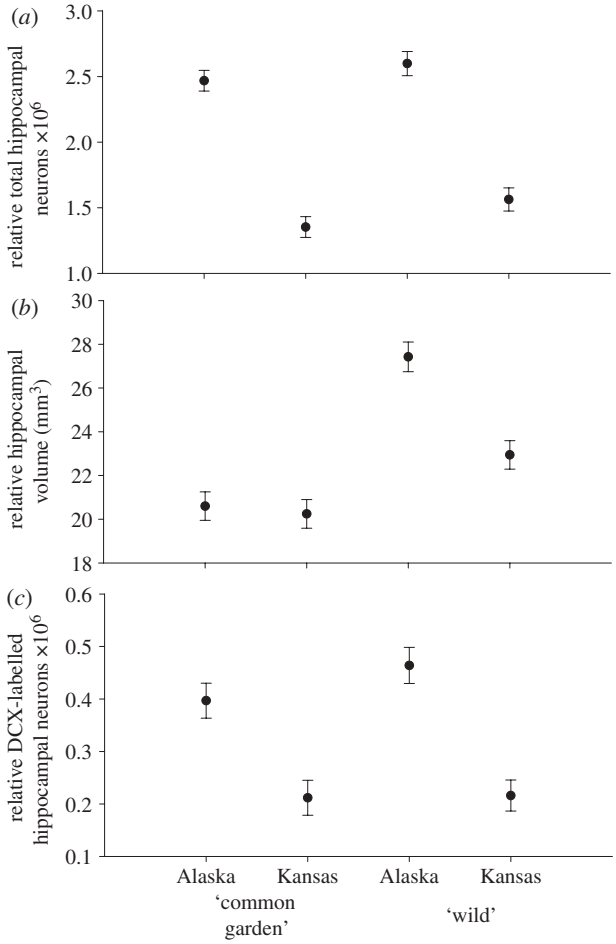

Figure 2. Black-capped chickadees originating from two very different climatic locations but hand-raised in the same laboratory environment expressed significantly different numbers of (*a*) hippocampal neurons, (*b*) similar hippocampal volumes, but (*c*) significantly different levels of neurogenesis. The 'common garden' birds were collected in 2009 and hand-raised in captivity. The data for the 'wild' populations were derived from previous studies at the same sites, but were collected in the autumn 2007 (Kansas) and 2008 (Alaska); data from Roth and co-workers [10–12]. Least-square means $\pm$ 1 s.e.m. are reported controlling for Te volume (total neuron number, hippocampal volume) and total neuron number (neurogenesis). The exclusion of covariates does not affect the results for total neuron number or neurogenesis, but does have an effect on Hp volume (see text for details).

(ii) *Associative learning spatial task*

Alaskan chickadees ($n = 12$) inspected fewer wells and achieved asymptote (i.e. had learned the task) significantly faster during the associative learning task than the birds from Kansas ($n = 12$; RMANOVA across trials 2–6; $F_{1,21} = 5.180$, $p = 0.033$; figure 1*c*). Both groups chose the rewarded well significantly more frequently than expected by random search (all p's < 0.001).

(b) *Morphological comparison between hand-raised populations*

(i) *Morphological measures*

Even though both groups of birds had experienced the same environment throughout virtually their entire lives, birds from Alaska ($n = 12$) had significantly more Hp neurons ($F_{1,21} = 102.118$, $p < 0.0001$; figure 2*a*) than those from Kansas ($n = 12$). The exclusion of covariates did not affect the results ($F_{1,22} = 137.783$, $p < 0.0001$).

There were, however, no differences between the groups in relative Hp volume ($F_{1,21} = 0.126$, $p = 0.726$; figure 2*b*), although Alaska birds had significantly larger absolute Hp volumes ($F_{1,22} = 4.499$, $p = 0.045$). This disparity between the relative and the absolute Hp volume comparisons were probably owing to Alaska birds having significantly larger Te volumes ($F_{1,22} = 6.084$, $p = 0.022$), even though they were smaller in body mass ($F_{1,22} = 26.534$, $p < 0.0001$).

(ii) *Doublecortin immunohistochemistry*

Birds from Alaska ($n = 12$) had significantly increased Hp neurogenesis (relative to the total number of Hp neurons) than those from Kansas ($n = 12$; $F_{1,21} = 8.360$, $p = 0.009$; figure 2*c*). The exclusion of covariates did not affect the results ($F_{1,22} = 69.448$, $p < 0.0001$).

(c) *Morphological comparison between hand-raised and wild-caught birds*

Interestingly, the estimates of total neuron numbers and neurogenesis in the Hp in both captive-reared populations were strikingly similar to those of wild birds collected previously from the same locations (figure 2*a*,*c*). When comparing the wild-caught (Alaska, $n = 12$; Kansas, $n = 13$) and common garden birds (Alaska, $n = 12$; Kansas, $n = 12$) in a single model, there was a significant effect of location ($F_{1,44} = 124.683$, $p < 0.0001$), but no effect of captivity ($F_{1,44} = 3.210$, $p = 0.080$), and no interaction effect ($F_{1,44} = 0.513$, $p = 0.477$) on neuron number (with Te volume as a covariate; figure 2*a*). Similarly, there was a significant effect of location ($F_{1,39} = 41.459$, $p < 0.0001$), but no effect of captivity ($F_{1,39} = 2.414$, $p = 0.128$), and no interaction effect ($F_{1,39} = 1.274$, $p = 0.266$) on neurogenesis (when controlling for total neuron number as a covariate; figure 2*c*). These results were similar when the data were analysed without covariates (total neurons—location: $F_{1,45} = 151.700$, $p < 0.0001$; captivity: $F_{1,45} = 4.100$, $p = 0.049$; interaction: $F_{1,45} = 0.600$, $p = 0.460$; neurogenesis—location: $F_{1,40} = 181.700$, $p < 0.0001$; captivity: $F_{1,40} = 3.000$, $p = 0.090$; interaction: $F_{1,40} = 1.200$, $p = 0.276$).

Our results strongly suggest that any effect of memory-based experiences or the environment on these two variables was negligible compared with the main mechanisms producing the differences between populations during very early development.

4. DISCUSSION

Overall, our results suggest that both genetic and environmental factors are at play in the creation of the patterns observed between the brain and the environment seen previously in wild black-capped chickadees. The mechanisms that produced the large-scale differences in the total number of Hp neurons and adult neurogenesis between these two populations seem not to be a function of experiences during late development or memory use that occurred during the autumn/winter caching season. Instead, these differences are probably inherited or produced at a very early stage of development prior to eye opening. Similarly, we also observed differences in caching behaviour and memory; hand-raised birds from the Alaska population cached more, had increased spatial memory accuracy and learned a spatial task more quickly than their hand-raised conspecifics from Kansas. By contrast, our results suggest a very strong effect of the environment (i.e. captivity) on Hp volume. There were no differences in Hp volume between the two hand-raised populations and both were significantly smaller than Hp volumes in their wild counterparts from previous studies [10,12]. Thus, it appears that Hp volume, unlike neuron number or Hp neurogenesis, is quite plastic and subject to environmental experiences.

Our findings regarding Hp neuron numbers are consistent with those reported in previous studies. For example, the difference in Hp neuron number between our two populations suggests that this trait is fairly fixed. This is consistent with studies that show no effects of captivity on Hp neuron number in adult wild birds (e.g. [9,31,32]). Thus, the available evidence suggests that even when animals experience different environments, the total number of neurons maintained in the brain may remain relatively stable, suggesting that this trait may be under genetic control.

However, our results regarding the consistency of neurogenesis levels stand in strong contrast to many previously published studies, including our own, which reported significant effects of the environment and experiences on Hp neurogenesis (e.g. [14,19,31]). We found that the large difference in doublecortin expression observed between our two previously studied wild populations was not different than those found in our birds that were hand-raised under identical environmental conditions. Although these results are different from many studies reporting a strong effect of environmental conditions on neurogenesis, they are at least somewhat consistent with more recent studies showing relatively high levels of heritable variation in neuron cell proliferation and survival among different strains of mice [33]. Although we cannot detangle this issue with our use of doublecortin, these studies as well as the results of this paper suggest that neuron production and survival rates may be under complex genetic control. Much more work is needed before we will fully understand the heritability of neurogenesis rates and other neural attributes.

It is clear that captivity can have an effect on brain morphology and processes (reviewed by Calisi & Bentley [34]). However, considering our current results with neuron numbers and neurogenesis, there may be a large difference between hand-raised, captive-reared animals and wild animals brought into captivity as adults. The latter may well experience long-term stress as a result of captivity, which could result in neurogenesis suppression [35], while the captive-reared animals may well perceive no such stressors in their environment. Anecdotally speaking, our captive birds in the laboratory showed no apparent signs of stress such as behavioural stereotypy, even after remaining in captivity for nearly 2 years (the siblings of those in this study; T. C. Roth, L. D. LaDage & V. V. Pravosudov 2009–2011, personal observation). This is in strong contrast to many of our other studies where we routinely see such stereotypy in wild birds held in captivity (T. C. Roth, L. D. LaDage & V. V. Pravosudov 2009–2011, personal observation). Although we acknowledge that our explanation is speculative, to our knowledge, this is the first comparison of brain morphology or levels of neurogenesis between captive-reared and wild animals from the same populations.

Future studies will hopefully attempt to address this unconventional result.

An alternative explanation for our results is that the genetic differences observed were mediated though differences in the behaviour (i.e. caching), which then produced the change in the brain. Our captive birds experienced a relatively enriched environment (e.g. they had the ability to cache food and retrieve caches at all times and were never isolated), although this level of environmental complexity and caching are certainly not comparable with wild levels [36]. It is possible, then, that these minor memory-based experiences in the laboratory were all that were necessary to produce Hp morphology and neurogenesis levels similar to wild counterparts, and any additional experiences, no matter how large, would have produced no significant additional effects (i.e. a step function response, *sensu* [24]). Still, this type of response requires that there be some pre-existing difference between the populations in the development of the Hp, genetic or otherwise, that simply required the trigger of only a few caching experiences to express. We note that although we observed small, yet significant differences in caching and memory in the testing rooms, we observed no differences in caching in the birds' cages. Moreover, the observed differences in the brain as mediated through a genetic difference in behaviour could not be produced via a proportional change in caching. For the observed difference in morphology to be a function of a genetically controlled difference in caching behaviour between the two populations, the relationship between caching intensity and brain morphology could not be proportional. The proportional difference in caching and memory use was much less than the morphological differences between the populations. Therefore, (i) any genetic effect on behaviour would have to be in place and inherently different for each population, (ii) the relationship between caching behaviour and brain morphology would need to be a step-function or something non-proportional, and (iii) the birds in both populations would have to have met their evolutionarily relevant caching requisite (assuming a step function) in the laboratory for the morphological differences to occur. While we cannot deny this possibility, the likelihood that these differences in the brain were owing to very small (relative to the wild), yet different (to produce the large differences in the brain) levels of caching seems low.

As our study was focused on the end result of the developmental process (i.e. birds during their first winter), we can only speculate that these differences may be owing to genetic differences in the rate of brain development. Differences in adult brain size and composition among avian taxa are known to be produced very early during development [37]. Whether these differences are apparent early on and remain throughout development or are produced later in life in chickadees will require further study. A very fruitful direction for future studies would be to attempt to understand at what point in development these two populations begin to differ and pinpoint the neural mechanisms of the deviation.

Unlike neuron number and neurogenesis, Hp volume was strongly affected by the environment—captivity, in the context of our design. We observed large differences in Hp volume between the two previously studied wild populations, but not in the captive-reared groups (figure 2*b*). In addition, captive-reared birds had significantly smaller Hp volumes compared with the wild birds sampled in the same populations (figure 2*b*). These results suggest that the volume of brain regions may be quite plastic [24,31,32,38,39]. It is possible and even likely that changes in the volume of a region that are independent of neuron numbers, as is the case here, may be the result of changes in dendritic structure of the neurons ([22], see also Cristol *et al.* [32]). With fewer connections and less neuronal arborization, the region may shrink more during fixation, resulting in smaller volume estimates. As changes to dendrites can occur very rapidly in response to environmental factors ([22] and references therein), we speculate that these changes in volume may represent an adaptive response [35] to the conditions experienced in captivity. These results also suggest that interpretations from the analysis of Hp volume should be viewed with caution. Although the volume of a brain region may be a relevant measure in some cases, the factors producing variation in this variable are complex and currently not well understood [22].

Our behavioural data suggest that, like Hp neural attributes, the drive to cache and the enhanced accuracy of spatial memory are probably produced by genetics or by factors occurring during very early stages of development, rather than a direct effect of memory usage or environment-related experiences. These results are consistent with the previous behavioural work in the system [3]. It is important to note, however, that both populations performed both memory tasks significantly better than random (figure 1*a*,*c*). In addition, the amount of caching was many orders of magnitude lower than that observed in wild parids (c.f. [36,40]; figure 1*b*). Therefore, even though we observed differences between the two captive groups, these minor, yet significant, differences are probably not the full explanation for the large differences in morphology between these groups. If that were the case, then we might expect significant differences between the captive groups, with neuron numbers and neurogenesis levels far lower than those observed in the wild. This was not the case.

In addition to differences in spatial memory and caching, we have also found striking differences in cognitive-based behaviours such as problem solving and the response to neophobia [23]. In those cases, the individuals from the Alaskan population showed significantly better problem-solving skills and a reduced neophobic response to a novel foraging situation, suggesting that selection may produce a suite of complex cognitive traits that could enhance survival in harsh conditions. Indeed, it is naive to predict that only one trait, e.g. spatial memory, will be the only or even main target of selection. Any trait whether behavioural, physiological or morphological that enhances survival and reproduction in harsh environments should potentially be adaptive. We contend that the complexity in the expression of various behaviours and their relationship to the complexity of brain morphology represents a key challenge for future studies of this kind and encourage more realistic predictions generated from the adaptive specialization hypothesis.

Theory and several previous studies suggest that the strong association between harsh environments and Hp volume, neuron number and neurogenesis, may be the result of selection for brain plasticity in different environments. We have eliminated this possibility as the sole explanation for the observed pattern in neuron numbers

and neurogenesis, but not for volumetric changes. We present, to our knowledge, the first evidence that differences in spatial memory, Hp neuron number and adult neurogenesis can be produced and maintained under identical laboratory conditions. This suggests a strong genetic or inherited component to the production and maintenance of these traits. However, our study also demonstrates that while these features seem to be largely fixed, some neurological features such as Hp volume may be quite plastic and heavily influenced by the reduced complexity of a captive environment. Although the role of experience is undoubtedly important in development and we cannot eliminate more complex effects in our study, variation in the environment does not fully explain the positive relationship between use, development and maintenance, and the neural mechanisms of cognition in some species. Ultimately, the relationship between variation in brain morphology and the environment is complex and a combination of both genetic and environmental factors. Future research will be needed to unravel this complex relationship to better understand the evolution of cognition.

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