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# SEX, ESTRADIOL, AND SPATIAL MEMORY IN A FOOD-CACHING CORVID

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## Abstract

Estrogens significantly impact spatial memory function in mammalian species. Songbirds express the estrogen synthetic enzyme aromatase at relatively high levels in the hippocampus and there is evidence from zebra finches that estrogens facilitate performance on spatial learning and/or memory tasks. It is unknown, however, whether estrogens influence hippocampal function in songbirds that naturally exhibit memory-intensive behaviors, such as cache recovery observed in many corvid species. To address this question, we examined the impact of estradiol on spatial memory in non-breeding Western scrub-jays, a species that routinely participates in food caching and retrieval in nature and in captivity. We also asked if there were sex differences in performance or responses to estradiol. Utilizing a combination of an aromatase inhibitor, fadrozole, with estradiol implants, we found that while overall cache recovery rates were unaffected by estradiol, several other indices of spatial memory, including searching efficiency and efficiency to retrieve the first item, were impaired in the presence of estradiol. In addition, males and females differed in some performance measures, although these differences appeared to be a consequence of the nature of the task as neither sex consistently out-performed the other. Overall, our data suggest that a sustained estradiol elevation in a food-caching bird impairs some, but not all, aspects of spatial memory on an innate behavioral task, at times in a sex-specific manner.

### Keywords

Spatial memory; estradiol; aromatase; scrub-jay; food caching

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## Introduction

In addition to their well-established role in the organization and activation of reproductive behaviors, sex steroid hormones also play a key role in modulating cognitive function. For example, estradiol and testosterone treatment have been shown to improve spatial memory in laboratory rodents (Daniel et al., 1997; Locklear & Kritzer, 2014; reviewed in Luine, 2014). Presumably, these effects are achieved through enhancement of neuronal connectivity and activity via estradiol's promotion of dendritic spine formation (Mukai et al., 2010; Romeo et al., 2004; Woolley, 1998) and long-term potentiation in the hippocampus (HP) (Córdoba Montoya & Carrer, 1997). However, estradiol's impact on spatial memory function is not always positive; instead, its influence depends on a variety of factors, including dose, duration, type of memory (i.e., working vs. reference memory), sex, and species differences in responsiveness (Galea et al., 2002; Lipatova et al., 2014; Luine, 2014; Woolley, 1998). In addition, hormonal mediation of spatial memory in non-model organisms, including those that do *not* undergo regular estrous cycles, such as birds, has only recently been explored (Bailey et al, 2013; Hodgson et al., 2008; Oberlander et al., 2004; Rensel et al., 2013).

Whereas circulating estradiol in females likely plays a role in spatial memory, local production of estradiol in the HP may mediate spatial memory in the absence of ovarian input in females or in males with little circulating estradiol. Although gonadectomy is used in many studies to examine the effects of sex steroid depletion on memory, this technique does not eliminate extra-gonadal sources of testosterone and estradiol such as those synthesized in the adrenals and brain, or other potential precursors in the full estrogen synthetic pathway (Schlinger et al., 2008).

In oscine songbirds, the enzyme aromatase is abundant in the HP in both males and females (Saldanha et al., 1998; Saldanha et al., 1999; Saldanha et al., 2000; Saldanha et al., 2004; Shen et al., 1995), and the brain expresses the upstream enzymes necessary for *de novo* estradiolsynthesis (London et al., 2006). In addition, when compared to breeding birds, aromatase activity persists or even increases in the HP of some non-breeding birds, in contrast with aromatase expression and activity in the hypothalamus which is generally elevated only during breeding, when circulating testosterone is elevated (Balthazart et al., 1990; Soma et al., 1999; Soma et al., 2003). Similarly, treatment of intact and castrated male and female zebra finches with testosterone has no effect on aromatase activity in the HP, although activity in the pre-optic area (POA) increases in response to testosterone (Vockel et al., 1990). These observations suggest that the preservation of local estradiol synthesis in the HP is important for behavior and/or physiology during non-breeding periods when extra-gonadal sources of androgens and estrogens may be important.

In the current study, we examined the nature of the relationship between estradiol and spatial memory in the Western scrub-jay (*Aphelocoma californica*; hereafter referred to as scrub-jay), a member of the corvid family (incuding jays, magpies, crows, and ravens) that routinely uses spatial memory to relocate food caches in nature. Scrub-jays not only possess impressive spatial learning and memory capabilities, but also display evidence of episodic memory (*what*, *where*, and *when*) with respect to food caches (Clayton & Dickinson, 1998).

In addition, the HP of the scrub-jay (relative to body mass) is one of the largest of corvids studied to date (Pravosudov & de Kort, 2006). The avian HP, which is situated on the dorsal surface of the brain, is homologous to the mammalian HP (Mayer et al., 2013). In addition, lesions to the songbird HP produce deficits in spatial learning and memory that are restored by HP transplants (Patel et al., 1997; Watanabe & Bischof, 2004). Based on their natural history and strong spatial capabilities, this species represents an excellent system in which to examine the interplay between estradiol, food caching, and spatial memory.

Scrub-jays create caches in which to hide food year-long; however, favored fresh foods such as insects and larvae are less abundant in the winter, leading jays to utilize previously stored, less-perishable food items to survive. Accordingly, caching behavior increases during the fall acorn mast, enabling individuals to store acorns for later retrieval in times of need (De Gange et al., 1989). It is plausible that HP production of estradiol facilitates this behavior when jays are in non-breeding condition, as a) previous research has demonstrated non-breeding behavioral dependence on local neuroestrogen production (e.g., aggression in song sparrows, *Melospiza melodia*; Soma et al., 2000) and b) we have biochemical and immunocytochemical evidence for aromatase in the non-breeding scrub-jay HP (Fig. 1). Additionally, there is evidence for both estrogen receptor (ER) expression and non-genomic effects of estradiol in the songbird HP (Gahr et al., 1993; Heimovics et al., 2012; Hodgson et al., 2008; Metzdorf et al., 1999). We therefore hypothesized that estradiol production, presumably acting within the HP, facilitates memory for cache locations in non-breeding scrub-jays.

To test the hypothesis that estradiol facilitates spatial memory in scrub-jays, we manipulated whole body, systemic estradiol levels in wild-caught, adult male and female scrub-jays and assessed spatial memory using a well-characterized cache and recovery testing paradigm (e.g., Clayton et al., 2006). We reduced endogenous estradiol through the use of an aromatase inhibitor, fadrozole (FAD). This ensured that sources of circulating estradiol (predicted to be inactive due to the non-breeding state) *as well as* brain estradiol were blocked (as in Cherrier et al., 2005; Hodosy et al., 2009; Moradpour et al., 2006; Rensel et al., 2013). In some of these individuals, estradiol levels were replaced or increased with a subcutaneous estradiol implant.

In addition to investigating the role of estradiol, we explicitly tested for sex differences in spatial learning and memory. Sex differences in spatial memory have been reported in studies of rodents (e.g., Jonasson, 2005; Sutcliffe et al., 2007), with males tending to perform with greater efficiency as compared to females. However, these differences may not arise from a decrement in memory in females, but rather from a sex difference in strategy employed to complete the task (Tropp and Markus, 2001; Williams and Meck, 1991). Interestingly, to our knowledge no previous assessment of spatial memory in scrub-jays has considered sex-specific effects. In light of our hypothesis that estradiol regulates spatial memory, we were particularly interested in investigating how sex interacted with our experimental treatment to affect cache recovery.

## Materials and Methods

Western scrub-jays were captured using peanut-baited Potter traps in multiple locations in Southern California and brought into captivity during the summer months (July-August). Appropriate state and federal collecting permits were obtained prior to capture. We caught a total of 19 individuals (independent young and adults) in 2013 (n = 7 females and 10 males). In addition, one individual was captured as a nestling and hand-raised in captivity during the summer of 2012 (a female); this individual participated in preliminary caching and retrieval tests prior to the current experiment. We confirmed that in the current experiment, behavioral measures obtained from this hand-raised individual were within the range of variation observed amongst the other individuals. Captive jays were housed in groups of 1-4 in rooftop aviaries and provided with *ad libitum* water and food (Roudybush Daily Maintenance Diet) supplemented with fresh fruits, vegetables, worms, and peanuts. The number of individuals housed per aviary varied because some individuals were less aggressive when caged with fewer conspecifics, whereas others were amenable to housing in larger groups. However, regardless of housing numbers, individuals were in auditory and visual contact with other jays at all times. DNA was extracted from whole blood using the Qiagen DNA Mini kit and sex was determined via polymerase chain reaction using the P2 and P8 primers described by Griffiths et al. (1998). We validated the use of these primers by including DNA from two individuals of a known breeding pair in preliminary reactions. In addition, PCR-determined sex was confirmed in individuals from another study by post-sacrifice gonadal inspection (n = 4 males and 2 females). Housing and maintenance of the jays, as well as all experimental procedures, were approved by the UCLA Chancellor's Animal Research Committee.

### Behavioral Testing

Individuals were subjected to a four-day series of cache and recovery tests, during which time individuals remained in the testing cage. These tests were similar to the paradigm utilized by Clayton et al. (e.g., Clayton & Dickinson, 1998). Testing took place during the months of October and November, when jays are non-breeding and exhibit caching behavior. Testing cages were located in one of three empty, interior enclosures within the rooftop aviaries, and subjects were in constant auditory contact and partial visual contact with those jays housed in adjacent aviaries (at a distance of 2.1 meters).

The general testing procedure was as follows: individuals were moved from the home cage into a testing cage (81.3 × 81.3 × 81.3cm constructed of 2.54cm wire mesh) on the afternoon prior to the first day of testing. The next morning, food was removed from the cage at 7AM to ensure that birds were sufficiently motivated to cache. Approximately 4 hours later, two caching trays filled with substrate (ground walnut shells) were placed adjacent to one another along the far wall of the cage. Each caching tray consisted of a white plastic ice cube tray with 16 wells attached to a clear Plexiglas base, with colored blocks arranged behind each tray to provide spatial cues. The orientation and color combination of blocks behind each caching tray was changed daily to ensure that unique spatial cues were provided on each day of testing. This species caches and retrieves regularly in the wild and requires very little motivation to perform in captivity (Clayton et al., 2006). Only one of the birds had

experience with the actual caching and retrieval test in the trays (the hand-raised bird from 2012); thus, to reduce neophobic responses and ensure that all birds were adequately habituated to the caching trays prior to testing, ice cube trays filled with ground walnut shells and colored blocks were placed into the birds' home cages at least several weeks prior to the initiation of the experiment. During the caching test, a bowl with 30 whole peanuts out of the shell was placed in the cage with the caching trays and the birds were given 15min to eat and cache peanuts. The peanuts and trays were then removed and the locations of each cache were recorded. To assess memory recall, each tray was emptied and replaced with new ground walnut shells 4 hours later, and peanuts were placed back in their original locations and covered immediately before returning the trays to the testing cage. Each bird had 15min to search, recover, and eat peanuts, after which the locations of remaining peanuts and/or re-cached peanuts were recorded. All trials were videotaped to avoid disturbing the birds during the trials. Birds remained food-deprived but with access to water during the 4-hour period between cache and retrieval tests, and each individual received one cache test and one retrieval test per day for 3 consecutive days.

On the fourth day, each individual again received a caching and a retrieval test, with one modification: during the cache test, a clear sheet of plexiglass (~0.64cm thick) covered one half of each caching tray (the side to be covered was determined randomly), allowing individuals access to only 50% of potential caching locations. During the retrieval test, the plexiglass sheets were removed, allowing birds to search in any location. We utilized this test (similar to Clayton & Dickinson, 1998) in order to elucidate gross-level differences in spatial memory discriminatory abilities.

### Estradiol Manipulation

Each individual was assigned to one of the following two treatment groups: blank implant + fadrozole (FAD; $n$ = 6 males and 4 females), or estradiol implant (E2) + FAD ($n$ = 6 males and 3 females). Individuals received two implants (both blank or E2), placed subcutaneously under the skin of the neck 5 days prior to the start of testing. Blank or E2-filled (Sigma) silastic implants were 15mm (males) or 12mm (females) in length (not including ~1mm of tubing on each side for adhesive). Implants for males were longer than for females to account for the higher body mass of males. An initial blood sample was taken from the brachial vein prior to implantation for determination of pre-manipulation circulating steroid levels. One day prior to the start of testing, individuals were moved to the testing cage and were given an oral dose of FAD dissolved in sugar water to ensure palatability (~17μg/g, a dose shown to effectively reduce aromatase expression in songbirds; Saldanha et al., 2004). We have found that oral dosing is a superior method of dosing with this compound (Rensel et al., 2013; Saldanha et al., 2004). The average body mass of males and females was used to calculate the dosage volume for each sex. Subsequent FAD doses of the same amount were given each morning for the duration of the experiment to ensure that endogenous E2 levels remained low (Saldanha et al., 2004). After the last retrieval test on the 4$^{th}$ day of behavioral testing, a blood sample was taken from the brachial vein for measurement of post-manipulation circulating steroid. Implants were then removed and individuals returned to their home cages. Blood samples were kept on ice until centrifugation in the laboratory at

10,000rpm for 10min to separate plasma from blood solids, and the plasma portion was frozen at −20C until assay (see below).

## Plasma Estradiol Assay

Preliminary validations with un-extracted, diluted plasma, as well as ether-extracted plasma, suggested significant interference with the enzyme immunoassay (EIA; Cayman Chemical Estradiol Kit). Therefore, we used a solid-phase extraction to extract the steroid from plasma samples (Chao et al., 2011; Newman et al., 2008). To validate this technique, we extracted a pool of scrub-jay plasma, then spiked the resulting sample (re-suspended in EIA assay buffer) with 2ng/ml radio-inert E2. We serially-diluted this sample to obtain multiple dilutions within the linear portion of the standard curve. Recovery of the radio-inert E2 in extracted plasma relative to a spiked buffer solution was in the acceptable range (80-120%) and subsequent dilutions were in agreement with one another (within 80-120%), indicating that interference was removed with the solid-phase extraction. Therefore, all plasma samples were run in the EIA at a final dilution of 1:6 (total plasma volume extracted = 25μl for females and 50μl for males; samples for males were run in both estradiol and testosterone assays). The limit of detectability of the assay (the value of the lowest standard on the curve) was 6.6pg/ml. The standard curve was logit-transformed prior to extrapolation of sample values, and the linearity of the curve verified ($R^2$ > 99%).

To perform the solid-phase extraction, 3ml Empore high performance extraction columns (C18 SD) were fitted to a PrepSep Vacuum Manifold (Fisher). Columns were conditioned with 250μl 100% methanol, followed by two rounds of 250μl deionized water. Samples were then added to the columns, followed by two rounds of 250μl deionized water. Finally, the sample was eluted into 5ml collection tubes using two rounds of 250μl 100% methanol. All steps were performed under vacuum pressure to facilitate movement through the columns. The resulting eluted samples were dried under a stream of air in a 50°C water bath, then concentrated with drops of dichloromethane down the sides of each tube (subsequently evaporated under a stream of air). Samples were re-suspended in assay buffer and subjected to EIA according to the manufacturer's instructions. Any samples with values below the lowest standard on the curve were assigned a numeric value of “zero.” The intra-assay CV (based on duplicate samples) was 2.5% and the inter-assay CV was 25% (based on a spiked buffer solution run in both assays).

## Plasma Testosterone Assay

Our study was designed to elucidate sex-specific effects of estradiol manipulation on spatial memory. However, application of FAD could inhibit negative feedback to the hypothalamic-pituitary-gonadal (H-P-G) axis, leading to elevated testosterone production. To determine whether this occurred, we measured plasma testosterone levels in males before and after experimental treatment. Due to limited availability of plasma, we conducted this test in males only, given that any effect of FAD on the H-P-G axis would be most likely to occur in males (whose circulating testosterone levels are likely higher than levels in females). An aliquot of the same plasma extracted for the estradiol assay was run in an enzyme immunoassay (Cayman Chemical Testosterone Kit) at a dilution of 1:6. The limit of

detectability was 3.9pg/ml, and all samples were above this limit (standard curve details were as above). Samples were run on one plate, with an intra-assay CV of 2.1%.

## Behavioral Analysis

The following variables were calculated from the video data for each bird on each day of testing as follows: number of items re-cached during retrieval, total number of searches during retrieval (multiple successive beak-swipes or probes in a given well were denoted as a single search; if an individual returned to that well after searching elsewhere, this was denoted as another search), the number of items retrieved, the number of 'rewarded' wells searched during retrieval (defined as wells in which a bird cached during the caching test), and the number of wells searched prior to finding the first item during retrieval. For data collected on day 4, we also calculated the number of searches during retrieval that occurred on the previously uncovered sides of the trays ("number searched on correct side"). All retrieval videos were scored by one observer blind to experimental treatment.

We also assessed whether the experimental treatment altered behaviors not directly related to spatial memory. To do so, we pseudo-randomly chose a subset of videos (balancing across sex and treatment group) from days 1-3 (6 males and 5 females) and day 4 (6 males and 6 females). We tabulated the time spent perching and the number of flights and hops that occurred during the retrieval test, not including any hops that occurred on the caching trays themselves. Videos from days 1-3 were scored by one individual, and videos from day 4 were scored by another, both blind to experimental treatment.

## Statistical Analysis

Data for days 1-3 were analyzed separately from day 4. For days 1-3, we used linear mixed models to assess the number of items cached, the number of items re-cached, and the total number of searches, and mixed binary logistic regression to assess the proportion of searches occurring in rewarded wells (those that contained a peanut cache after the cache test), the proportion of items recovered, and the proportion of searches occurring prior to finding the first item. The independent variables in each of these analyses were sex, day (1-3), treatment (FAD + Blank, hereafter referred to as -E2, or FAD + E2, hereafter referred to as +E2), the sex by day interaction, and the day by treatment interaction. We did not include the sex by treatment interaction in any models from days 1-3 because only 2 females in the +E2 group participated in the caching and retrieval tests. Where appropriate, we included covariates such as the number of items cached (for the analysis of number of items re-cached) and the number of wells containing a cached item (for all other recovery tests). To maintain the simplicity of the models, we only included these covariates if they significantly predicted variation in the response variable when run separately in a mixed model regression. Results for covariates are presented in Tables 1-2 but not discussed in the text. For all models assessing days 1-3, bird identity was included as a random factor to control for repeated sampling, and we utilized a Sattherthwaite degrees of freedom correction for low or variable sample sizes.

To analyze the results from day 4, we used the same models described above, with several modifications: 1) day was not included in the models; 2) the sex by treatment interaction

was included in all models because sample sizes on this day were sufficient; 3) we included the dependent variable “proportion of searches occurring on the correct side” (the uncovered side of each tray) and 4) we used either general linear models or binary logistic regression (using the likelihood ratio test) because there was no repeated sampling of individuals.

We also sought to identify patterns in search behavior: i.e., did individuals cluster their searches around cache locations even if they did not find the items, and did they show stronger memory for those cache locations that contained multiple items? To assess these questions, for each search that an individual undertook during retrieval, we quantified the distance of the search from the nearest cache location (i.e., a search in an adjacent well would be given a score of “1”), as well as the number of items that had been previously cached in that location. Since the two caching trays were situated adjacent to one another, we calculated overall distance of a search from the nearest cache location, considering the two trays as one long, 32-well tray. As there were two rows on each caching tray, a search that was located diagonal to a correct location was given a distance of “1”, the same designation as that used for an adjacent well search.

To analyze these data, we conducted a binary logistic regression that accounted for the maximum possible search distance from a cache site, using a mixed design for days 1-3 with bird ID as a random factor. We included sex, treatment, and day (for days 1-3 only) as fixed factors, as well as the sex by day interaction (days 1-3 only), day by treatment interaction (days 1-3 only), and sex by treatment interaction (day 4 only). We also included a continuous variable, “number of items in the nearest well”, to address whether or not searches were clustered near wells in which more items had been cached. To account for variation in the number of searches that each individual performed on a given day, the model was weighted by the total number of searches performed (ln transformed). No Satterthwaite corrections were used due to large sample sizes.

Since our estradiol manipulation was systemic and not restricted to the HP, it is possible that outcomes other than spatial memory were affected by the experimental treatments. To assess whether the manipulations non-specifically influenced activity and/or motivation, time spent perching and number of flights/hops were both assessed using general linear models, with sex and treatment as fixed factors.

Plasma estradiol levels were mostly too low to be detected by the assay (see results below). Because so many values were undetectable (except for in those individuals treated with estradiol), we did not analyze the raw E2 data, but instead assessed the probability that a sample was detectable (on-curve) using a mixed binary logistic model. Treatment group (-E2 or +E2), time (pre or post-implantation), sex, and the associated two-way interactions were included in the model, along with bird identity as a random term. Two samples (out of 38) were missing from this analysis. Plasma testosterone levels in males were analyzed using a mixed model with time (pre or post-implantation) and treatment group as fixed effects. One sample was missing from this analysis (n = 12 males; 23 total samples).

All statistics were run in SPSS 22, and data presented are estimated means generated from the models ± 1 S.E. Non-significant interaction terms were removed in backwards stepwise

fashion to achieve final models. Effect sizes are reported for general linear model effects discussed in the text (partial eta squared ($\eta^2$) for model effects and Cohen's d for pairwise comparisons).

## Results

### Plasma Estradiol & Testosterone

The probability that a given sample was detectable in the assay (i.e., on the curve) was influenced by the treatment by time interaction ($F_{1,31}$ = 4.5; P = 0.04): birds in the -E2 group had equally low probabilities of plasma E2 being on the curve before and after implantation ($F_{1,31}$ = 0.1; P = 0.78), while the probability increased in birds in the +E2 group after implantation ($F_{1,31}$ = 19.4; P < 0.001; Fig. 2). The probability of a sample being detectable was not influenced by overall treatment ($F_{1,31}$ = 2.3; P = 0.14), time (pre vs. post; $F_{1,31}$ = 3.0; P = 0.09), sex ($F_{1,31}$ = 0.3; P = 0.56), the treatment by sex interaction ($F_{1,30}$ = 0.0; P = 0.998), or the time by sex interaction ($F_{1,29}$ = 0.0; P = 0.997). These results verify that 1) un-manipulated circulating E2 was low to undetectable as would be expected during the non-breeding season and 2) our E2 implantation protocol significantly elevated circulating levels.

Testosterone was detected in the plasma of all samples from males (12 pre and 11 post-implantation) and levels were unaffected by time (pre or post-implantation; $F_{1,20}$= 3.7; P = 0.07), treatment ($F_{1,20}$ = 3.5; P = 0.08), or the interaction between time and treatment ($F_{1,19}$ = 0.1; P = 0.79; Fig.2). Therefore treatment with FAD did not induce a change in circulating testosterone in males. While we cannot be certain that FAD treatment had no effect on the H-P-G axis of females, these data are consistent with the idea that the H-P-G axis of scrub-jays is not responsive during this time of year.

### Caching Behavior

**Days 1-3—**The number of items cached did not differ between the sexes ($F_{1,16}$ = 0.6; P = 0.45), treatments ($F_{1,16}$ = 0.0; P = 0.93), across days (although there was a trend towards increased caching from days 1 to 3; $F_{2,36}$ = 3.0; P = 0.06), or as a function of the sex by day or day by treatment interactions ($F_{2,32}$ = 0.1; P = 0.87 and $F_{2,34}$ = 0.9; P = 0.42, respectively). Similarly, the probability of caching did not depend on treatment ($F_{1,15}$ = 0.1; P = 0.80), sex ($F_{1,14}$ = 1.4; P = 0.25), day ($F_{2,52}$ = 2.0; P = 0.14), or the treatment by day or day by sex interactions ($F_{2,50}$ = 0.4; P = 0.67 and $F_{2,48}$ = 0.3; P = 0.73, respectively).

**Day 4—**On day 4 of testing when ½ of each caching tray was covered with clear plexiglass to prevent access, there was no difference in the number of items cached between males and females ($F_{1,15}$ = 0.3; P = 0.58; $\eta^2$ = 0.02) or between treatments ($F_{1,15}$ = 0.9; P = 0.35; $\eta^2$ = 0.06). However, there was a significant sex by treatment interaction ($F_{1,15}$ = 5.1; P = 0.04; $\eta^2$ = 0.25): in males, -E2 and +E2 birds did not differ in the number of items cached ($F_{1,15}$ = 1.1; P = 0.3; d = −0.61) whereas in females, there was a trend for individuals in the -E2 group to cache more items than females in the +E2 group ($F_{1,15}$ = 4.1; P = 0.06; d = −1.55). The probability of caching was not tested because only 2 birds failed to cache on day 4, a male in the -E2 group, and a female in the -E2 group.

## Retrieval Behavior

**Days 1-3—**The proportion of searches occurring prior to the first successful recovery depended on day ($F_{2,29}$ = 4.2; P = 0.025) as well as the sex by day interaction and the treatment by day interaction ($F_{2,29}$ = 4.8; P = 0.015 and $F_{2,29}$ = 5.0; P = 0.013, respectively). Specifically, males conducted proportionally fewer searches prior to finding the first item than females on days 2 and 3 ($F_{1,28}$ = 4.2; P = 0.049 and $F_{1,17}$ = 5.0; P = 0.039, respectively), but not on day 1 ($F_{1,29}$ = 0.3; P = 0.605 Fig. 3a). There was no difference between treatment groups in proportion of searches occurring prior to the first recovery on days 1, 2, or 3, although -E2 birds tended to need fewer searches before acquiring the first reward on day 3 than +E2 birds ($F_{1,23}$ = 3.9; P = 0.06; days 1 and 2 P > 0.2). When the same interaction was analyzed according to treatment group, there was a significant effect of day in -E2 birds ($F_{2,29}$ = 6.1; P = 0.006): birds needed proportionally fewer searches to find the first item on day 3 than on days 1 (P = 0.02) or 2 (P = 0.006; days 1 and 2 did not differ; P = 0.53), suggesting that these birds improved in retrieval efficiency over time. No change was seen in the +E2 group ($F_{2,29}$ = 0.1; P = 0.94; Fig. 3b).

The total number of searches undertaken during the retrieval test, the number of re-caching events, the proportion of searches occurring in rewarded wells, and the proportion of items recovered did not depend on sex, treatment, day, or the interactions between these factors (see Table 1 for full statistical results).

**Day 4—**Individuals in the -E2 group searched proportionally more rewarded wells than individuals in the +E2 group ($X^2$ = 9.1; P = 0.003; Fig. 4) on day 4. In addition, females required proportionally fewer searches to recover the first item than males ($X^2$ = 10.2; P = 0.001; Fig. 5a) as did individuals in the -E2 group relative to the +E2 group ($X^2$ = 5.1; P = 0.02; Fig. 5b). Finally, the proportion of searches made on the “correct sides” or previously uncovered sides of the caching trays was predicted by the sex by treatment interaction ($X^2$ = 4.9; P = 0.03). Males in the two treatment groups did not differ in their search behavior on the uncovered vs. covered sides (P = 0.60), but among females, -E2 individuals searched proportionally *more* wells on the correct side than those in the +E2 group (P = 0.007; Fig. 6). The total number of searches undertaken during the retrieval test, the proportion of items recovered, and the number of re-caching events did not vary with respect to sex, treatment, or the interaction between sex and treatment (see Table 2 for full statistical results).

## Search Behavior

**Days 1-3—**The mean search distance from the nearest cache on days 1-3 was approximately 2 wells. Males and females did not differ in their search distances, nor did the two estradiol treatment groups ($F_{1,829}$ = 0.5; P = 0.48 and $F_{1,829}$ = 0.1; P = 0.76, respectively); additionally, there was no effect of day ($F_{2,829}$ = 1.9; P = 0.15). There was, however, a significant interaction between day and treatment group ($F_{2,829}$= 22.6; P < 0.001) as well as an interaction between sex and day ($F_{2,829}$ = 22.6; P < 0.001). In the -E2 group, there was a significant effect of day ($F_{2,829}$= 5.2; P = 0.005): individuals searched closer to reward sites on day 3 than on days 1 (P = 0.01) or 2 (P 0.003; Fig. 7), while days 1 and 2 did not differ (P = 0.245). In the +E2 group, the converse pattern emerged ($F_{2,829}$ = 4.6; P = 0.011): individuals searched *farther* from reward sites on day 3 than on days 1 (P = 0.003) or

2 (P = 0.017; days 1 and 2 did not differ, P = 0.22; Fig. 7). In females, there was no effect of day ($F_{2,829}$ = 2.8; P = 0.06), but there was a significant effect in males ($F_{2,829}$ = 3.7; P = 0.025): males searched farther from reward sites on day 3 than on day 1 (P = 0.01; all other pairwise comparisons P > 0.05). Finally, across days and treatment groups, search distances were closer to reward sites when those sites contained more items ($F_{1,829}$ = 11.4; P = 0.001).

**Day 4—**The mean search distance for all birds from the nearest cache on day 4 was approximately 2 wells. Sex and treatment had no effect on the distance of a given search from the reward ($F_{1,336}$ = 1.1; P = 0.29 and $F_{1,336}$ = 0.3; P = 0.57, respectively), but search distances were closer to the reward when the nearest reward site contained more items ($F_{1,336}$ = 11.4; P = 0.001). There was no interaction between sex and treatment ($F_{1,335}$ = 0.2; P = 0.66).

### Non-Spatial Behaviors

**Days 1-3—**For all birds, time spent perching during the retrieval test did not depend on sex ($F_{1,8}$ = 0.2; P = 0.67) or treatment ($F_{1,8}$ = 0.1; P = 0.79; the sex by treatment interaction was not analyzed due to insufficient sample size in female +E2 group). Similarly, the number of flights and hops was unaffected by treatment or sex ($F_{1,8}$ = 1.9; P = 0.20 and $F_{1,8}$ = 0.2; P = 0.65, respectively).

**Day 4—**On the fourth day of testing, time spent perching by the jays did not depend on sex ($F_{1,9}$ = 0.2; P = 0.64; $\eta^2$ = 0.03), treatment ($F_{1,9}$ = 0.1; P = 0.75; $\eta^2$ = 0.01), nor the interaction between sex and treatment ($F_{1,8}$ = 0.1; P = 0.80; $\eta^2$ = 0.01). However, +E2 males exhibited more hops and flights during the retrieval trial than –E2 males ($F_{1,8}$ = 11.9; P = 0.009; d = 2.17), although this relationship did not exist in females ($F_{1,8}$ = 0.1; P = 0.80; d = −0.39; sex by treatment interaction: $F_{1,8}$ = 6.9; P = 0.03; $\eta^2$ = 0.46). Notably, none of the retrieval variables assessed showed a male-specific effect of treatment; therefore it is unlikely that the effect of estradiol on male activity biased the spatial memory data. Sex and treatment alone did not predict number of hops and flights, although there was a trend towards more hops and flights in +E2 individuals ($F_{1,8}$ = 2.1; P = 0.18; $\eta^2$ = 0.21and $F_{1,8}$ = 5.1; P = 0.06; $\eta^2$ = 0.39, respectively).

## Discussion

This investigation tested the hypothesis that estradiol facilitates memory for cache locations in Western scrub-jays. This is, to our knowledge, the first such study undertaken in a food-caching songbird, and is unique among spatial memory studies in the use of an ecologically-relevant behavioral task. In addition, we asked if there were sex differences in memory for cache locations and whether any effects of estradiol were related to sex. While measures of activity and motivation as well as overall cache recovery rates were no different in the presence or absence of E2, we found that, contrary to our predictions, several measures of retrieval behavior were impaired in the presence of estradiol. Additionally, we observed several sex differences in indices of spatial memory function, both across and between treatment groups.

Several lines of evidence confirm that the captive scrub-jays were indeed in non-breeding condition and that our treatments effectively increased and/or decreased estrogen levels and did so with limited side effects. Under these natural non-breeding photoperiod conditions, and prior to the start of testing, estradiol was undetectable in the circulation of both male and female scrub-jays, confirming minimal gonadal steroidogenesis at this time. Testosterone was detected in male samples, but these levels were considerably below what is detected in temperate-breeding male songbirds (e.g., Moore et al., 2002; Smith et al., 1997). We ensured that all sources of estradiol were blocked using daily oral dosing of an aromatase inhibitor, FAD, a dose that has proven effective previously at blocking songbird HP aromatase (Saldanha et al., 2004). Given the absence of peripheral estradiol production, we assume that treatment with FAD only functionally eliminated central, but not peripheral sources. Moreover, the absence of an effect of FAD on plasma testosterone levels in males emphasizes the lack of an influence on the photorefractory HPG axis, and therefore limited unexpected gonadal androgen synthesis.

We have found that aromatase is present in the non-breeding scrub-jay brain and report, in separate studies (Ellis, Rensel, and Schlinger, in prep), its presence in regions highly conserved across other avian species, such as the pre-optic area, ventromedial nucleus of the hypothalamus and in the homologue of the mammalian amygdala, the nucleus taeniae (Schlinger and Balthazart, 2013). In addition, aromatase is conspicuously present in regions found in other oscine songbirds, including the HP and caudo-medial nidopallium (NCM) (Saldanha et al., 2013). Although at present we have not investigated ER expression in the scrub-jay HP, other studies have documented ER expression in the songbird brain (Arnold et al., 1976; Saldanha & Coomaralingam, 2005), including the HP (Gahr et al., 1993; Gahr, 2001; Hodgson et al., 2008; Metzdorf et al., 1999), as well as expression during the non-breeding season (Fusani et al., 2000; Wacker et al., 2010). Because ER and aromatase are not limited to the HP, it is possible that our systemic treatments non-specifically influenced jay behavior via effects occurring outside the HP. Our experimental design does not allow us to rule out this possibility. However, we found only limited effects of FAD and subsequent replacement with E2 on measures of general activity, specifically hops and flights in the cage and time spent perching. In addition, there was no treatment effect on caching activity itself, arguing that the estradiol manipulation did not influence the motivation to participate in this behavior. Taken together, these data suggest that the documented effects of estradiol removal and replacement were not non-specific in nature and likely restricted to spatial memory.

Over the first three days of testing, males improved in efficiency to retrieve the first item while females did not, suggesting that males adjusted more readily to the caching and retrieval task (surprisingly, however, males searched on average *farther* from reward sites on day 3 than on days 1 or 2). However, when birds were asked to search for caches on day 4 in trays that had been 50% covered during the caching phase, females were more efficient than males in finding the first item. One possibility is that males and females use different strategies to search for and uncover caches that benefit one or the other sex under different contexts. It appears that females found the more gross-level memory task of day 4 easier than males, who were in turn better focused on the more detail-oriented task of days 1-3.

Additionally, on day 4, females in the -E2 group displayed enhanced memory for the correct, uncovered side of the trays relative to +E2 birds, an effect that was not seen in males. This suggests that the strategy used by females was more sensitive to effects of estradiol than the strategy employed by males. These sex-specific effects on spatial memory retrieval are intriguing, especially since so few studies of estradiol and spatial memory in birds consider sex (but see Hodgson et al., 2008) and sex differences in spatial memory of jays have not been previously addressed.

It is unknown why males and females differed in retrieval performance or responses to estradiol. The sex differences in retrieval performance could point to differences in neural circuitry underlying spatial memory function, perhaps differentially organized by hormones early in development and/or activated by sex-specific hormonal fluctuations (Luine, 2014). Further studies will assess sex differences in HP aromatase, estrogen receptor (ER), androgen receptor (AR), and the other components of the steroid synthetic pathway as a means to uncover the mechanism underlying sex differences in scrub-jay performance.

A particularly intriguing result of our study is that estradiol impairs several aspects of spatial memory in scrub-jays. However, while we observed decrements in spatial memory function in +E2-treated birds, there were no overall differences between treatment groups in retrieval efficacy over any of the days of testing. This is not entirely surprising, given that the 15min retrieval period likely enabled individuals to search and retrieve all cached peanuts regardless of experience or minor memory impairments. Thus, while estradiol appears to negatively impact fine-grain aspects of memory such as search efficiency and efficacy, individuals in the +E2 group were able to compensate for any deficits within the 15min time frame of the retrieval period. Specifically, we found that +E2-treated jays exhibited decrements in the proportion of searches directed to the rewarded, or cached-in, locations, the proportion of searches on the side of the tray that was not previously covered, and efficiency in retrieving the first item. Many of these effects were day or sex-specific, suggesting that the effects of estradiol removal and replacement are modulated by experience and sex-specific factors. For example, -E2-treated birds improved in first retrieval efficiency between days 1 to 3, while those in the +E2 group did not. Similarly, –E2-treated birds searched closer to rewarded sites on day 3 than on days 1 or 2, while +E2-treated birds searched closer to rewards on day 1 but ended up searching farther from rewards on day 3.

These impairments of memory function in the presence of estradiol are difficult to reconcile with the obvious presence of aromatase in the jay HP. It is possible that due to the low levels of circulating testosterone in the non-breeding season, little or no estradiol is naturally produced in the HP, ensuring that optimal memory function is retained. Nevertheless, it is important to reconcile differing results between scrub-jays and zebra finches, in which estradiol appears to facilitate spatial memory retrieval (Bailey et al., 2013; Rensel et al., 2013). Several possible explanations could account for the negative impact of estradiol on scrub-jay spatial memory as well as the species differences noted above. First, the underlying architecture supporting spatial memory formation, consolidation, and retrieval may differ between the cache recovery task used here and the maze navigation task used in zebra finches (as well as in many mammalian studies (e.g., Bowman et al., 2002; Daniel et

al., 1997). Second, neuroestrogens may undergo somewhat rapid local fluctuations, as has been noted in the zebra finch NCM (Remage-Healey et al., 2008, 2012) according to the specific phase of the spatial memory task. In this manner, estradiol could promote one phase of the spatial memory process, such as memory retrieval, but inhibit another phase such as memory formation. Indeed, our group found previously in female zebra finches that inhibition of estrogen synthesis with FAD impaired spatial memory retrieval, but enhanced spatial memory acquisition (Rensel et al., 2013). Note, however, that in a study in male zebra finches, application of an aromatase inhibitor directly to the HP led to impairment of *both* acquisition and retention of spatial memory (Bailey et al., 2013). Thus, the chronic manipulation of estradiol, as performed in these studies, may mask effects on spatial memory processes that would be observed with acute and targeted neurohormone manipulations.

A third possibility is that the dosage of estradiol applied or the relative ratio of local estradiol to testosterone may have significantly affected our results. For example, non-breeding aggression appears to be dependent on local estradiol production in the brain in song sparrows (Soma et al., 2000); however, induction of higher than normal estradiol levels through gonadotropin-inhibitory hormone or estradiol infusions in the quail brain leads to a *decrease* in aggression (Ubuka & Tsutsui, 2014). These studies point to a negative effect of estradiol, depending on the context and degree of elevation. It is also possible that by inhibiting aromatase, FAD may have indirectly increased local levels of testosterone in our study. While testosterone is known to exert positive effects on spatial memory through its conversion to estradiol by aromatase, testosterone (or its non-aromatizable androgenic metabolite 5α-dihydrotestosterone; DHT) may adversely affect spatial memory retrieval by binding to androgen receptors. In support of this hypothesis, a study in which male zebra finches were treated with DHT demonstrated impaired spatial memory formation in these individuals as compared to those treated with aromatizable testosterone, estradiol, or control implants (Oberlander et al., 2004).

It is important to note that while many studies across vertebrate taxa highlight the benefits of estradiol for spatial memory, there are indeed a number of mammalian studies that document negative effects or, in some cases, dose-specific effects of estradiol. In addition to the factors discussed above, the presence of contradictory findings could be the result of any number of differences in study design, such as whether or not stress or reward is involved (Bowman et al., 2002; Locklear & Kritzer, 2014), sex of the animal tested (Galea et al., 2002), whether other hormones such as progesterone are manipulated (Chesler & Juraska, 2000), the dose and/or type of estrogen applied (Holmes et al., 2002; Luine, 2014; Sinopoli et al., 2006; Ubuka & Tsutsui, 2014), the mode and duration of application (Luine, 2014), and unique life history characteristics of the species such as cyclicity of normal estradiol secretion (Lipatova et al., 2014) or lack thereof (e.g., birds). Another often overlooked factor is the fact that gonadectomy only removes gonadal sources of estradiol or testosterone, while extra-gonadal sources such as the brain may continue to produce estradiol through local synthesis (Luine, 2014). The HP of mammals, in addition to birds, expresses aromatase, indicating that it has estrogen-synthetic capacities (Roselli, 2013). Only a handful of mammalian studies have investigated the potential for extra-gonadal estradiol

synthesis to impact spatial memory, however. Of these, two studies have utilized aromatase inhibition to block whole-body estradiol synthesis in male rats. Both studies found positive effects of estrogen removal on spatial memory function (Alejandre-Gomez et al., 2007; Moradpour et al., 2006), results that appear to be in agreement with those of the current study. Interestingly, using FAD and E2 treatments in non-breeding European starlings (*Sturnus vulgaris*), Calisi et al. (2013) found evidence for reduced auditory learning in the presence of estradiol, suggesting that non-spatial aspects of learning and memory can be impacted by estradiol during the non-breeding season. Future experiments should manipulate and further test the role of region-specific estradiol production in the HP and other brain areas involved in cognition.

In summary, our data suggest that estradiol impairs spatial memory formation and/or retrieval during the non-breeding season in a food-caching corvid (when replaced in FAD-treated individuals using implants). We found that while overall recovery rates were identical between estradiol-removal and replacement groups, birds in the replacement group exhibited subtle decrements to aspects of spatial memory such as efficiency and improvement over time. The results of this study contribute to a small but growing body of literature in both mammals and birds that seeks to understand the mechanisms by which steroid hormones synthesized in the brain mediate spatial memory. In addition, this is the first study to assess the impact of estrogens on spatial memory in a food-caching bird, a common model system for studying cognition and memory. We encourage other groups to conduct studies in ecologically relevant systems such as the scrub-jay, utilizing naturally-occurring behaviors that have long been subject to the processes of natural selection. In this way we can continue to elucidate the processes by which complex cognition, reproduction, and behavior interact to produce species that are suited to their natural environments.

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## Highlights

- We tested the role of estradiol in spatial memory function in Western scrub-jays
- Estradiol supplementation induced decrements in cache retrieval indices
- Overall cache recovery rates were unaffected by estradiol
- Some effects were sex or experience-dependent

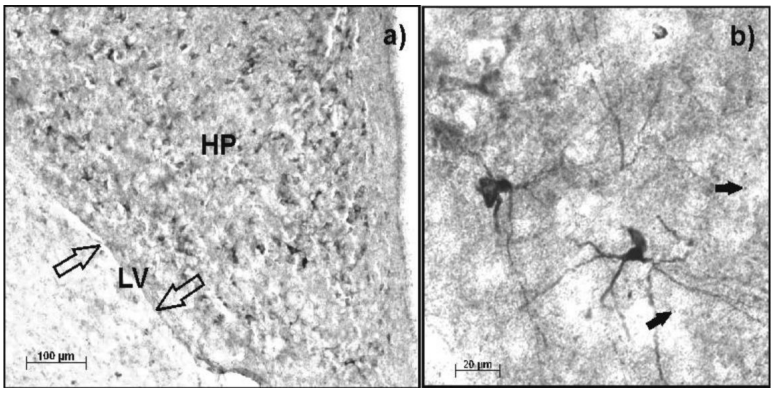


**Fig. 1.**

Low (a) and high (b) power photomicrographs showing localization of aromatase staining in the adult scrub-jay HP. A) An abundance of stained soma and fibers are found in the HP, while relatively low levels of staining are found in the adjacent telencephalon. Open arrows point to the lateral ventricle (LV). B) At high power, the degree of somal and fiber staining in the HP is easily observed, as well as staining of punctate structures (dark arrows). Immunohistochemistry was performed as follows: after perfusion with 4% paraformaldehyde, brains were sectioned at 40 microns and stored in antifreeze at −20°C. Immunohistochemistry was performed following established protocols (Ellis and Riters, 2013; Heimovics and Riters, 2005) with the following changes: because brains were fixed with formaldehyde, no sodium borohydride was used and associated washes were eliminated. Anti-aromatase primary antibody (Saldanha et al., 2000) was used at 1:5000 in 0.1M phosphate buffered saline (PBS). Secondary was biotinylated goat anti-rabbit (1:1000; Vector Labs). After incubating in AB solution, sections were washed twice for five minutes each in PBS, then washed three times in 0.175M sodium acetate (Hoffman et al., 2001). After treatment with diaminobenzidine (DAB), sections were washed in 0.175 sodium acetate 2× for five minutes before a final 5min wash in PBS.

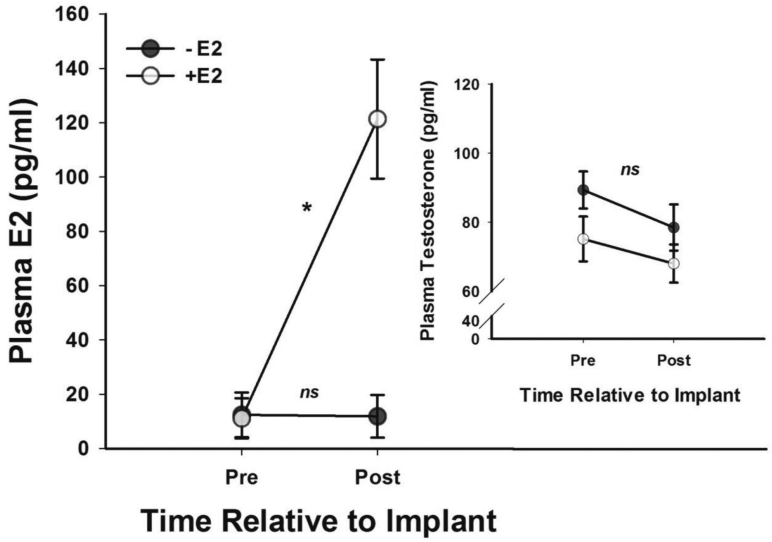


**Fig. 2.**

Plasma estradiol levels before and after experimental treatment with FAD and an implant (blank or E2; n = 12 males and 7 females). Inset: plasma testosterone levels in males before and after experimental treatment (n = 12 males)

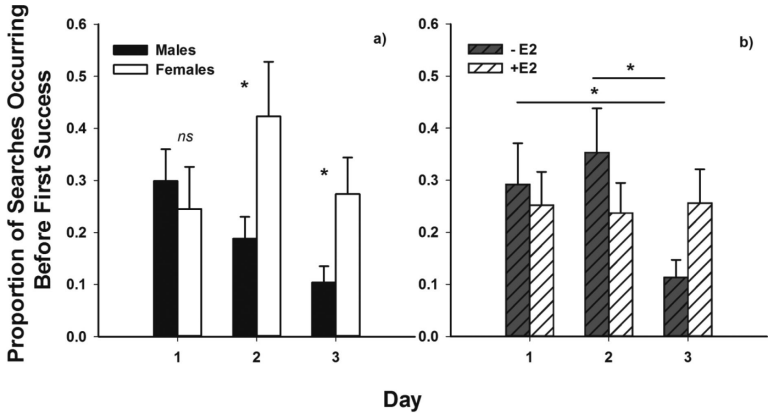


**Fig. 3.**

The proportion of searches occurring prior to retrieval of the first peanut across the 3 days of testing between a) males and females and b) treatment groups (* indicates $P < 0.05$). A lower value indicates that fewer wells were searched before success.

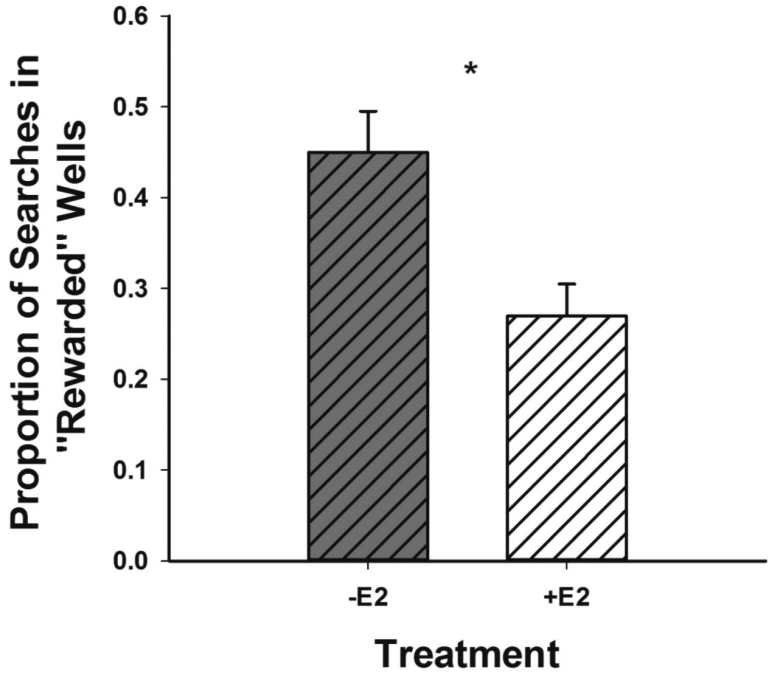


**Fig. 4.**

The proportion of searches undertaken in “rewarded” wells (aka wells containing a cached peanut at the start of the retrieval test) on day 4 of testing, according to experimental treatment (* indicates $P < 0.05$).

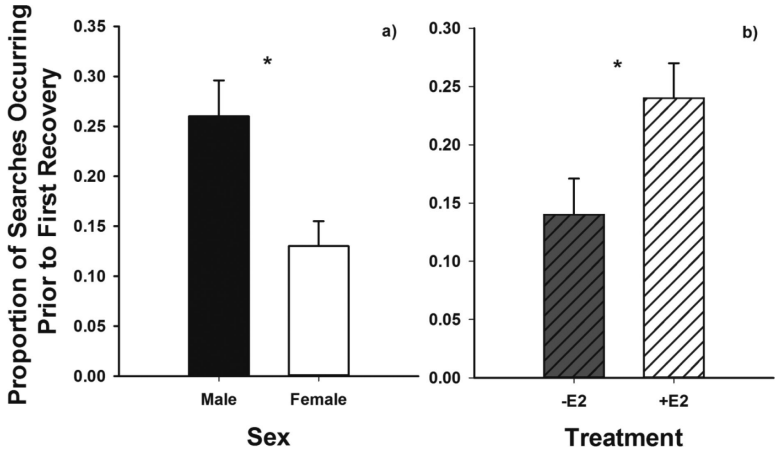


**Fig. 5.**

The proportion of searches occurring prior to retrieval of the first peanut on day 4 of testing according to a) sex and b) experimental treatment. A lower value indicates that fewer wells were searched before success (* indicates $P < 0.05$).

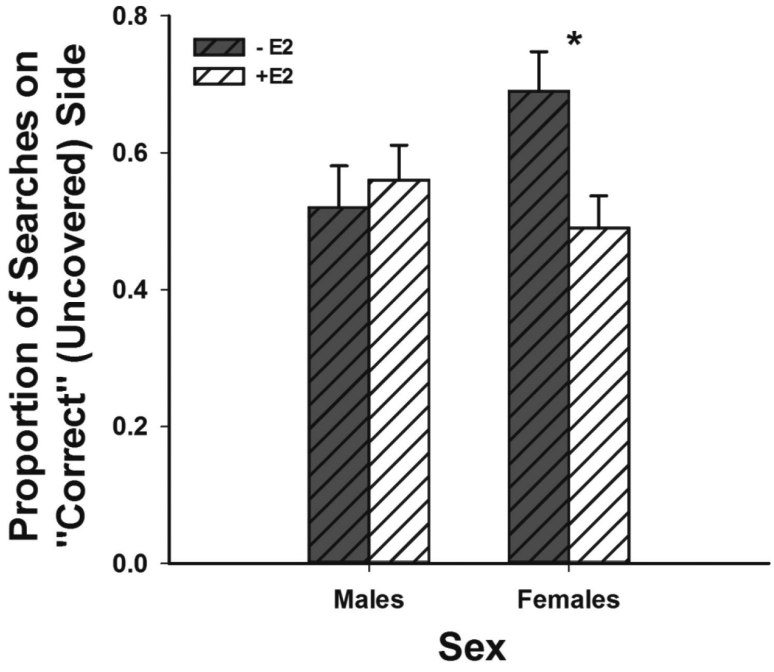


**Fig. 6.**

The proportion of searches undertaken on the “correct” (aka previously uncovered) side of the caching trays during the retrieval test according to experimental treatment and sex (* indicates $P < 0.05$).

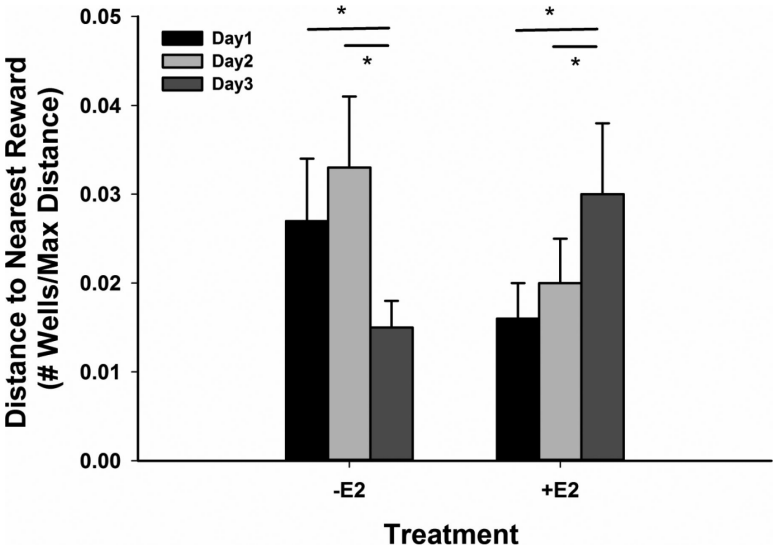


**Fig. 7.**

Mean distance of individual searches from the nearest cached peanut during the retrieval period (expressed as a proportion of the maximum possible search distance from a cache site), according to day and experimental treatment. A smaller distance indicates that searching was localized closer to the peanut cache (* indicates $P < 0.05$).

## Table 1

Retrieval Variables Assessed on Days 1-3.

| | F or $X^2$ | P-value |
|---|---|---|
| ***Number of Searches (n = 41):*** | | |
| *Sex* | *0.4* | *0.56* |
| *Treatment* | *0.5* | *0.49* |
| *Day* | *0.1* | *0.92* |
| *Sex * Day* | *0.8* | *0.45* |
| *Treatment * Day* | *0.5* | *0.62* |
| ***Proportion Recovered (n = 40):*** | | |
| *Sex* | *0.0* | *0.85* |
| *Treatment* | *0.3* | *0.62* |
| *Day* | *2.1* | *0.14* |
| *Sex * Day* | *0.6* | *0.55* |
| *Treatment * Day* | *0.8* | *0.47* |
| ***[c]Number of rewarded wells*** | ***14.3*** | ***0.001*** |
| ***Proportion of Searches in Rewarded Wells (n = 40):*** | | |
| *Sex* | *0.9* | *0.37* |
| *Treatment* | *0.8* | *0.40* |
| *Day* | *0.7* | *0.50* |
| *Sex * Day* | *1.6* | *0.23* |
| *Treatment * Day* | *0.5* | *0.63* |
| ***[c]Number of rewarded wells*** | ***47.7*** | ***< 0.001*** |
| ***Proportion of Searches Prior to 1st Recovery (n = 39):*** | | |
| *Sex* | *2.8* | *0.12* |
| *Treatment* | *0.0* | *0.85* |
| ***Day*** | ***4.2*** | ***0.025*** |
| ***Sex * Day*** | ***4.8*** | ***0.015*** |
| ***Treatment * Day*** | ***5.0*** | ***0.013*** |
| ***[c]Number of rewarded wells*** | ***9.2*** | ***0.005*** |
| ***Number Re-cached (n = 41):*** | | |
| *Sex* | *1.3* | *0.27* |
| *Treatment* | *0.1* | *0.78* |
| *Day* | *0.2* | *0.81* |
| *Sex * Day* | *2.4* | *0.12* |
| *Treatment * Day* | *0.6* | *0.55* |
| ***[c]Number of items cached*** | ***28.9*** | ***< 0.001*** |

Factors in bold are significant at the $P < 0.05$ level.

[c] denotes a covariate included in the model.

## Table 2

Retrieval Variables Assessed on Day 4.

| | F or $X^2$ | P-value |
|---|---|---|
| ***Number of Searches (n = 17):*** | | |
| *Sex* | *2.1* | *0.17* |
| *Treatment* | *0.5* | *0.47* |
| *Sex * Treatment* | *0.5* | *0.51* |
| ***Proportion Recovered (n = 17):*** | | |
| *Sex* | *3.5* | *0.06* |
| *Treatment* | *0.0* | *0.94* |
| *Sex * Treatment* | *0.3* | *0.56* |
| ***[c]Number of rewarded wells*** | ***12.2*** | ***< 0.001*** |
| ***Proportion of Searches in Rewarded Wells (n = 17):*** | | |
| *Sex* | *0.0* | *0.86* |
| ***Treatment*** | ***9.1*** | ***0.003*** |
| *Sex * Treatment* | *3.3* | *0.07* |
| ***[c]Number of rewarded wells*** | ***22.2*** | ***< 0.001*** |
| ***Proportion of Searches Prior to 1st Recovery (n = 17):*** | | |
| ***Sex*** | ***10.2*** | ***0.001*** |
| ***Treatment*** | ***5.1*** | ***0.02*** |
| *Sex * Treatment* | *2.0* | *0.16* |
| ***Number Re-cached (n - 17):*** | | |
| *Sex* | *2.4* | *0.14* |
| *Treatment* | *2.2* | *0.16* |
| *Sex * Treatment* | *0.6* | *0.44* |
| ***[c]Number of items cached*** | ***5.2*** | ***0.04*** |
| ***Proportion of Searches on "Correct" Side (n = 17):*** | | |
| *Sex* | *0.7* | *0.40* |
| *Treatment* | *2.2* | *0.14* |
| ***Sex * Treatment*** | ***4.9*** | ***0.03*** |

Factors in bold are significant at the $P < 0.05$ level.

[c] denotes a covariate included in the model.