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ORIGINAL PAPER

Influence of sex steroid hormones on spatial memory in a songbird

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Abstract In mammals, sex steroid hormones influence spatial learning and memory abilities but there are few data regarding such effects in birds. We investigated whether non-invasive sex steroid hormone treatment would affect spatial memory task performance of great tits (*Parus major*). For five consecutive days, birds were fed wax moth larvae injected with either 80 μg testosterone or 80 μg estradiol carried in peanut oil immediately prior to behavioral testing. During the 5 days prior to and the 5 days following hormone treatment, birds were fed vehicle-injected larvae. Both hormone manipulations resulted in an elevation of circulating hormone levels within 5 min of larva ingestion. This elevation was sustained for at least 30 min but had no short-term (<1 day) effect on spatial memory performance. However, performance tended to increase during the first 5 days of vehicle treatment and during both sex steroid treatments whereas it decreased during the 5 days of vehicle treatment following either hormone treatment. These results suggest that both hormones led to some improvement in spatial memory that declined once treatment ended. The great tit hippocampus was found to express androgen and estrogen receptors which would provide a direct site of sex steroid action.

Keywords Androgen receptor · Estrogen receptor · Hippocampus · Memory · Songbird

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Introduction

The hippocampus is essential for spatial memory performance in songbirds (Hampton and Shettleworth 1996; Patel et al. 1997; Sherry and Vaccarino 1989), as it is in mammals, suggesting that mechanisms of hippocampal-dependent function may be conserved. In mammals, sex steroid hormones are known to influence spatial learning and memory abilities (e.g., Gouchie and Kimura 1991; Roof 1993; Williams and Meck 1991) through their effects on the hippocampus (e.g., Frye et al. 2004; Packard et al. 1996). Androgens can improve cognitive performance either by acting directly via the reduced metabolite dihydrotestosterone (DHT; Frye et al. 2004) or by aromatization into estrogens by aromatase (*estrogen-synthase*, Hojo et al. 2004). Estrogens (originating from the gonads, or synthesized in the hippocampus de novo) produce changes in hippocampal-dependent memory via their action on estrogen receptor isoforms alpha and beta (Prange-Kiel et al. 2003).

In the classical model of sex steroid action, biological activity is thought to occur predominantly through binding to intracellular receptors, which are members of the nuclear receptor superfamily. These then interact with specific nucleotide sequences to alter gene expression. As this

process takes at least 30 min, immediate behavioral effects cannot occur. In contrast, non-genomic or rapid actions of steroids take place within minutes and there is mounting evidence for such effects from mammals, birds and fish (e.g., Frye 2001; Moore and Evans 1999; Naghdi and Asadollahi 2004; Remage-Healey and Bass 2004; Balthazart et al. 2006; Foradori et al. 2008). The precise mechanism of action has yet to be described, but the behavioral changes must be driven by non-genomic actions on neural circuits.

The effects of the sex steroids on spatial learning and memory in birds have received little attention, but it is plausible that their effects are similar to those observed in mammals since the songbird hippocampus is a site of high aromatase and reductase expression and activity (Saldanha et al. 1998; Schlinger 1997; Gahr 2001). There is evidence for effects of neural aromatization on songbird spatial memory as estrogen, but not testosterone, delivered over 10 days accelerates the rate of spatial memory acquisition (Oberlander et al. 2004).

The objective of the present study was to determine the short-term effects of sex steroids on spatial memory in songbirds. We employed a non-invasive method whereby steroid hormones were injected into wax moth larvae and immediately fed to the birds. Rapid and short-term effects on behavior have been reported using glucocorticoids via this method (Breuner et al. 1998; Breuner and Wingfield 2000). In this study, adult great tits (*Parus major*) were treated with testosterone, estradiol or vehicle immediately prior to being tested on a hippocampal-dependent spatial memory task (spatial delayed non-matching-to-sample, DNMTS; Biegler et al. 2001). Each treatment was repeated for five consecutive days so that both short- and medium-term effects on cognition could be examined. If, as in mammals, testosterone and estrogen act on spatial cognition abilities, we predicted that these sex steroids would lead to enhanced performance on the DNMTS task. A secondary goal of this study was to verify the hippocampal expression of androgen and estrogen receptor (alpha and beta) mRNA to confirm sites for genomic action of sex steroids on spatial cognition. Cell-surface sex steroid receptors remain poorly understood (Balthazart et al. 2006; Wehling and Losel 2006) and therefore were not studied.

Materials and methods

Animals

Eight male and 16 female adult great tits (all wild-caught in deciduous woodland, Edinburgh, UK) were housed individually in wire-mesh cages (77 × 44 × 44 cm). They were provided daily with fresh water and ad libitum insectivorous bird food mixture (Orlux, Sunring Cooke, Greasbrough, Rotherham, UK), supplemented by peanuts, sunflower seeds and wax-moth larvae. Birds were maintained on a 9 h light:15 h dark cycle (lights on 08:00; lights off 17:00) at temperatures of 14–16°C. Short days were used to ensure that endogenous levels of sex steroids were basal. Birds were food deprived at 08:00 each morning and behavioral testing was performed from 10:00 until 16:00. Each testing session lasted approximately 40 min for each individual bird and food was replaced at the end of the session. The order in which the birds were tested each day was randomized, and birds tested later in the day were provided with a peanut once an hour until testing. All procedures were carried out under licence in accordance with guidelines defined by the United Kingdom Home Office.

Behavioral measurement

Birds were trained to perform a delayed-non-matching-to-sample task presented on computer-controlled touch screen. The images used in the task were generated on Intasolve touch screens (21 cm × 28 cm), driven by Acorn A7000+ computers, placed on trolleys that could be moved to the front of a bird's cage. A sliding door in the front of the bird's cage allowed the bird to have direct access to the touch screen. A standard rat-pellet dispenser (Campden Instruments 442 Pellet Dispenser) was used to deliver food rewards (ca. 20 mg peanut piece) via a plastic tube onto a small tray inside the bird's cage beside the touch screen. Further details are provided by Biegler et al. (2001).

Behavioral training and testing

All birds were trained to peck at two white squares (2 cm × 2 cm) on a computer-controlled touch screen (the sample phase). Each image disappeared after a peck was directed at them. Once both images had been pecked there was a retention interval (RI) of 1 s before the birds were presented again with two squares (the test phase): one of which was in one of the locations used in the sample phase while the second was in a new location. Birds were rewarded with a small piece of peanut for pecking the square in the new location. All choices were followed immediately by the onset of a 90 s inter-trial interval.

All birds were trained to a criterion of at least 70% correct choices averaged over three consecutive days. In total there were 20 trials (each consisting of a sample/test cycle) in each daily testing session and the 20 trials typically took a bird approximately 40 min to complete. To reduce variability in success rate between birds, once the birds reached the criterion we began a titration procedure to adjust the RI for each bird to either make the task easier (shorter RI) or more difficult (longer RI). In the titration procedure, the RI

was increased by 0.3 s in the trial following a correct choice, but in a trial following an error, the RI was decreased by 0.7 s. The minimum RI was 1 s. This titration phase lasted for 400 trials (in blocks of 20 trials, one block per day). The RIs achieved by each bird on each of the last five blocks of testing were averaged and used as the RI for that bird in the hormone manipulation phase of the experiment.

Hormone manipulation

During the hormone manipulation phase of the experiment, all birds were tested for 25 consecutive days, receiving 20 trials per day. The steroid administration protocol was as follows: 5 days of vehicle (control), 5 days of either testosterone or estradiol, 5 days of vehicle, 5 days of either testosterone or estradiol, and 5 days of vehicle. A treated wax-moth larva was fed to the bird immediately before behavioral testing each day. Vehicle treatment consisted of a wax-moth larva injected with 10 µl of peanut oil. Steroid treatments consisted of a wax-moth larva injected with 10 µl of testosterone (8 mg/ml; Sigma) or 10 µl of estradiol (8 mg/ml; ß-Estradiol (1, 3, 5 (10)-Estratriene-3, 17ß-diol); Sigma) in peanut oil, respectively. The order of hormone treatment was allocated randomly and was counterbalanced such that 12 birds received the testosterone treatment first and the estrogen treatment second, and the order of hormone treatment was reversed for the remaining 12 birds.

Hormone measurement

To quantify the levels of hormone circulating in the blood following treatment, birds were bled following ingestion of a hormone-injected larva or a vehicle-injected larva. Birds were bled at three time points approximately two weeks apart: (A) 5 min after ingestion of a hormone-injected larva, (B) 30 min after ingestion of a hormone-injected larva, and (C) 5 min after ingestion of a vehicle-injected larva. The order in which treatments A, B, and C were administered was randomized. Eight birds (five females and three males) were fed a testosterone-injected larva at two time points and a vehicle-injected larva at the third and all three blood samples were measured for testosterone levels. Eight different birds (five females and three males) were fed estradiol-injected larvae or a vehicle-injected larva and all three blood samples were measured for estradiol levels.

A 100 µl blood sample was taken from each bird by puncturing the alar wing vein with a 25G needle and collecting the blood into heparinized microhematocrit tubes. Blood was kept on ice until it was centrifuged (14,000 rpm for 10 min). The plasma was stored at −20°C until transported frozen on dry ice to the University of Washington where levels of testosterone and estradiol were measured by radioimmunoassay after partial purification on diatomaceous earth/glycol columns. Plasma was extracted in redistilled dichloromethane and dried under a stream of nitrogen. Extracts were re-dissolved in 10% redistilled ethyl acetate in iso-octane and transferred to the columns. Steroid fractions were eluted by increasing concentrations of ethyl acetate in iso-octane as a function of polarity (also see Ball and Wingfield 1987; Wingfield et al. 1991).

Data analysis

For each hormone, we tested for an effect of treatment on plasma levels of steroids using the GLM procedure in SAS software (SAS Institute, SAS Publishing, Cary, NC) with bird and treatment (5 min after ingestion of a hormone-injected larva, 30 min after ingestion of a hormone-injected larva, and 5 min after ingestion of a vehicle-injected larva) as independent factors. Steroid levels were log-transformed prior to analysis.

We tested for differences in spatial memory performance with hormone treatment using the MIXED procedure in SAS, with the total number of correct choices (out of 20) made by a bird on a given day as the dependent variable, and sex, treatment and other terms as effects in the model, as described below. Repeated measures analysis was performed using the REPEATED statement with bird as the subject.

In situ hybridization histochemistry for androgen receptor and estrogen receptor alpha and beta mRNA expression

A 759 bp fragment encoding the zebra finch androgen receptor cDNA was sub-cloned into pGEM-7Zf. Antisense and sense riboprobes were generated by in vitro transcription, in the presence of 35S-UTP, with SP6- and T7-RNA polymerase after plasmid linearization with *Eco*RI or *Hind*III, respectively. Zebra finch estrogen receptor alpha sense and antisense riboprobes were generated by sub-cloning a 926 bp fragment encoding the estrogen receptor alpha cDNA into pGEM-7Zf. In vitro transcription in the presence of 35S-UTP, with T7- and SP6-RNA polymerase was performed after plasmid linearization with *Bam*HI or *Eco*RI, respectively. For estrogen receptor beta, a 427 bp fragment cloned into pGEM-7Zf was linearized with *Eco*RI and *Hind*III and in vitro transcription was performed in the presence of 35S-UTP, with SP6- and T7—RNA polymerase to create sense and antisense probes, respectively. All three clones have high homology with the canary and chicken suggesting that the cloned fragments should be well conserved, at least within passerines. The clones were generously provided by Drs M. Gahr and R. Metzdorf; Max-Planck-Institute of Behavioural Physiology, Germany (Gahr and Metzdorf 1997).

To examine the distribution of androgen and estrogen receptor alpha and beta mRNA expression in the tit hippocampus, two male great tits were killed and their brains removed and frozen immediately on dry ice. Whole brains were sectioned coronally at 15 μm on a cryostat and thaw mounted onto clean polysine coated glass microscope slides and stored at −70°C. Every fifth section was separately mounted and stained with Toluidine-Blue (Sigma, Poole, UK) to locate regions of interest. Slide-mounted sections containing the hippocampus from each bird were thawed to room temperature and immersed in 4% paraformaldehyde solution for 10 min then rinsed in PBS before treatment with 0.3% triethanolamine/acetic anhydride. The slides were then rinsed in PBS and dehydrated through a series of graded ethanol. Sections were then incubated at 50°C with prehybridization solution for 2 h, and hybridised with the 35S-labelled antisense or sense riboprobe directed against androgen receptor or estrogen receptor alpha in a solution mixed with 50% formamide. The probe was applied to each section at a concentration of 10^6 cpm per slide in 200 μl hybridization solution for 18 h at 55°C in a humidified chamber. Post hybridization washes consisted of 3 × 5 min washes in 2× saline–sodium citrate (SSC). Sections were then incubated in a 30 μg/ml ribonuclease A (RNase-A) solution for 1 h at 37°C, followed by a 30 min rinse in 2× SSC at room temperature followed by stringency washes in 0.1× SSC at 50°C for 90 min, then 2 × 60 min rinses at room temperature. Test assays were used to determine the optimal wash temperature for these probes. Tissue was then dehydrated in a graded series of ethanol containing 300 mM ammonium acetate. The hybridization signal was visualised at the cellular level by dipping the slides in autoradiographic emulsion (Ilford 135 5053). Slides were air dried, and stored with desiccant at 4°C for 8 weeks before being developed (Kodak D19), counterstained with haematoxylin and eosin and finally coverslipped with DPX mountant. Slides were examined with a light microscope, under bright field illumination.

Specificity of hybridization signal

Control procedures for the antisense androgen or estrogen receptor probe included hybridization of sections with the sense riboprobe or pre-treatment with RNase-A prior to hybridization with the antisense riboprobe, conducted under identical conditions to those for the antisense probe.

Results

Hormone administration

The effect of treatment on circulating hormone levels was assessed by comparing the plasma levels of the steroid 5 and 30 min after ingestion of a hormone-injected larva with those measured five minutes after ingestion of a vehicle-injected larva. Plasma testosterone levels increased significantly following testosterone treatment ($F_{2,14} = 19.03$, $P < 0.0001$), with testosterone levels remaining high 30 min after ingestion of the testosterone-injected larva (Fig. 1). Estradiol administration tended to increase the level of plasma estradiol, although not significantly ($F_{2,14} = 3.50$, $P = 0.06$; Fig. 1).

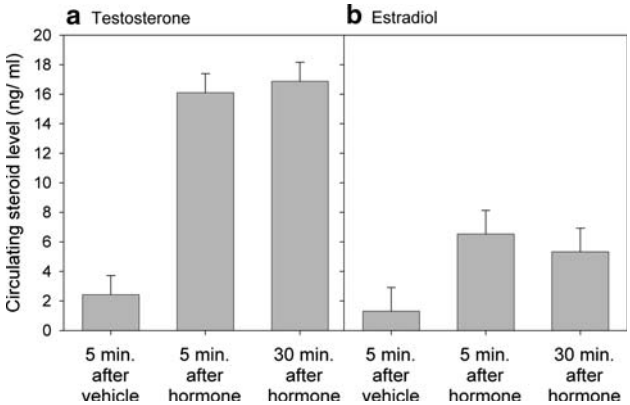

Fig. 1 Effect of testosterone and estradiol treatment on plasma levels of steroids in great tits. **a** Testosterone levels 5 min after ingestion of a vehicle-injected larva, 5 minutes after ingestion of a testosterone-injected larva, or 30 min after ingestion of a testosterone-injected larva ($N = 8$ birds). **b** Estradiol levels 5 min after ingestion of a vehicle-injected larva, 5 min after ingestion of an estradiol-injected larva, or 30 min after ingestion of an estradiol-injected larva ($N = 8$ birds). Steroid levels were log-transformed prior to analysis and means and standard errors were reverse-transformed for clarity

Spatial memory performance

To assess short-term effects of hormonal treatment on spatial memory performance, we analyzed the total number of correct choices (out of 20) made by a bird on a given day in response to treatment, including sex and the sex-by-treatment interaction as factors, with day of the experiment (i.e., 1–25) as a linear covariate and bird as a subject. The sex-by-treatment interaction term was not significant ($F_{2,44} = 0.36$, $P = 0.70$) and so was removed from the model. Neither sex ($F_{1,22} = 3.01$, $P = 0.10$) nor treatment ($F_{2,46} = 0.70$, $P = 0.50$) had a significant effect on the number of correct choices, but the number of correct choices increased very slightly over the course of the experiment (0.04 ± 0.01 correct choices out of 20/day, or one correct choice over the entire period of the experiment; $F_{1,568} = 13.10$, $P < 0.001$).

To assess medium-term effects of hormonal treatment, a model similar to that described above was used, but an additional term was included, day within treatment (i.e., 1–5), to examine changes in performance throughout the 5 days of testing on a given treatment. For this analysis we

distinguished between three types of control treatment: the first 5 days of vehicle (prior to any hormone treatment), the 5 days of vehicle following estradiol treatment and the 5 days of vehicle following testosterone treatment. The interaction between treatment and day within treatment was significant ($F_{4,560} = 3.45$, $P < 0.01$), i.e., the changes in performance over the 5 days of each treatment differed significantly between treatments. Performance tended to increase during the first 5 days of vehicle treatment and during both the estradiol and testosterone treatments, although none of these increases was significant ($P > 0.15$ in all cases). In contrast, performance decreased significantly during the 5 days of vehicle treatment following either hormone treatment ($P < 0.05$ in both cases; Fig. 2). This effect was not the result of deterioration in performance towards the end of the experiment. In our experimental design, hormone manipulations took place on days 6–10 and 16–20, and the 5 days following hormone treatment were days 11–15 and 21–25 (see "Hormone manipulation").

Hippocampal androgen and estrogen receptor mRNA distribution

All regions of the hippocampus of the great tit expressed mRNA for androgen receptor and estrogen receptors alpha and beta (Fig. 3). There was no detectable hybridization signal with the sense probe, or following RNase-A pretreatment (Fig. 3d).

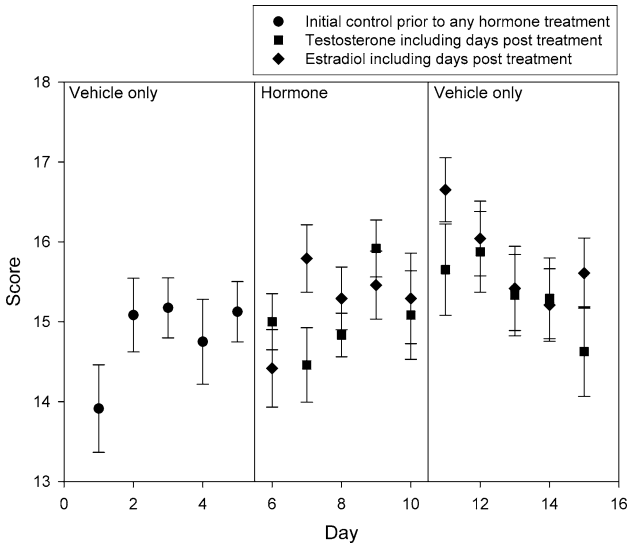

Fig. 2 Changes in performance in a spatial memory task prior to, during and following treatment with testosterone or estradiol. The *left panel* shows performance over the first 5 days of the experiment, prior to any hormone treatment. The *middle panel* shows performance during the 5 days of treatment with testosterone (*squares*) or estradiol (*diamonds*), which occurred on days 6–10 and days 16–20 of the experiment (half of the birds were treated with testosterone, followed by vehicle treatment, followed by estradiol treatment, followed by vehicle treatment, whereas the order of the steroid treatments was reversed for the other birds). The *right panel* shows performance during the 5 days of treatment with vehicle following treatment with testosterone (*squares*) or estradiol (*diamonds*), which occurred on days 11–15 and days 21–25 of the experiment. *Error bars* denote standard errors

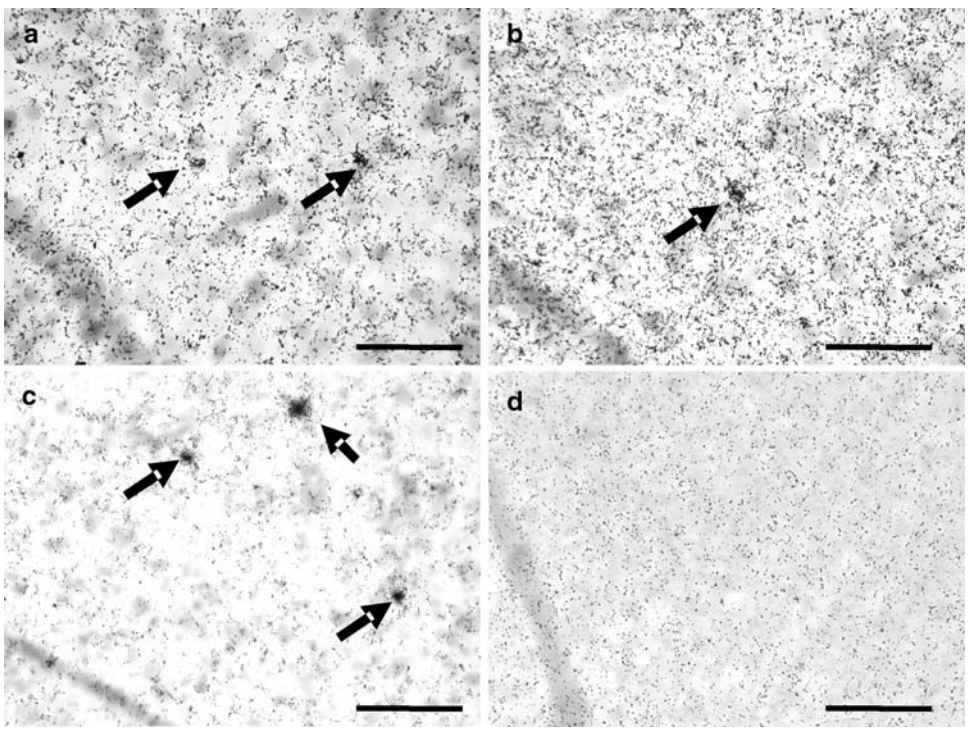

Fig. 3 Photomicrograph examples illustrating androgen receptor (**a**) and estrogen receptor alpha (**b**) and beta (**c**) mRNA expression in the hippocampus of the great tit. Photomicrograph (**d**) illustrates lack of hybridization signal in the hippocampus using the sense riboprobe for androgen receptor. *Arrows* point to cells expressing receptor mRNA. *Scale bar* 50 μm

Discussion

We have demonstrated that plasma sex steroid levels can be significantly elevated in great tits by feeding them a wax-moth larva injected with a sex steroid hormone. Ingestion of a testosterone-treated larva elevated plasma levels of testosterone (measured by radioimmunoassay) approximately sevenfold from at least 5–30 min after ingestion, compared with control levels. Plasma estradiol levels were also increased after ingestion of an estradiol-treated larva, although this effect was marginally nonsignificant. Given that treatment elevated steroid levels for at least 30 min, hormone levels are predicted to have been elevated for the entire duration of the spatial memory task (approximately 40 min). Treatment with testosterone and estradiol did not appear to have any short-term effects on the birds' performance on a spatial delayed-non-matching-to-sample task. However, the changes in performance throughout the 5 days of each treatment indicate slight medium-term effects of testosterone and estradiol on spatial performance, where spatial memory tended to improve during treatment and declined once treatment ended. This was not due to a general increase in performance due to practice across the experiment as there was a decrease in performance during vehicle treatment following either hormone treatment. Given that we observed a medium-term response to sex steroids with this task but were unable to detect a short-term response, our data do not provide support for a fast-acting, non-genomic mechanism of sex steroids on avian spatial memory (Wehling 1995).

The medium-term effects of estradiol and testosterone observed in the present study are not surprising given that the mechanisms allowing sex steroids to affect spatial cognition are clearly in place in the great tit, as they are in other songbirds (Gahr et al. 1993; Saldanha et al. 1999). Previously it has been shown that there is considerable aromatase activity in the telencephalon of the great tit (Silverin et al. 2000). Using in situ hybridization, we found high levels of expression in the hippocampus of great tits indicating sex steroid receptor sensitivity, which is consistent with the presence of sex steroid receptors in the hippocampus of other songbirds (Gahr et al. 1993; Metzdorf et al. 1999; Soma et al. 1999) and of rodents (Kerr et al. 1995; Weiland et al. 1997).

We know of only one other study showing an effect of exogenous sex steroids on cognitive abilities in songbirds. Estradiol, delivered by implant for 10 days to castrated male zebra finches, was correlated with greater improvement in a spatial memory task compared with implants of testosterone, dihydrotestosterone or an empty control (Oberlander et al. 2004). In both this previous study and the present study, it seems unlikely that the lack of a major effect was because the birds' had reached ceiling performance such that hormone treatment could not improve it yet further. As can be seen from Fig. 2, the birds in our experiment were not close to perfect performance.

Rapid effects of steroids on avian behavior have been observed in white-crowned sparrows (*Zonotrichia leucophrys gambelli*). For instance, corticosterone increases perch-hopping within 15 min of ingestion, a response that occurred concurrently with steroid elevation in a dose-dependent manner (Breuner et al. 1998; Breuner and Wingfield 2000). Birds with intermediate levels of corticosterone were more active while birds with high levels did not differ from controls (Breuner et al. 1998; Breuner and Wingfield 2000). This inverted U-shaped behavioral response to corticosterone manipulation leaves open the possibility that the hormone doses in our present study were too high to elicit an immediate response, and that lower levels of sex steroids may cause a rapid effect on spatial cognition. However, the levels were not so high that they had no effect at all: both sex steroids tended to increase spatial cognition performance across the 5 days of treatment, and there was a significant decrease in performance once treatment ended.

In conclusion, we have established that testosterone and estradiol may be effectively delivered to songbirds via an oral route to provide a non-invasive means of rapidly raising blood testosterone and estrogen levels. Treatment with both testosterone and estradiol affected performance on a test of spatial memory over the course of a few days, but we found no evidence of non-genomic, short-term (<1 day) effects.

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