Published in final edited form as:

Dev Neurobiol. 2015 March ; 75(3): 271–286. doi:10.1002/dneu.22228.

Dynamic variation in forebrain estradiol levels during song learning

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Abstract

Estrogens shape brain circuits during development, and the capacity to synthesize estrogens locally has consequences for both sexual differentiation and the acute modulation of circuits during early learning. A recently-optimized method to detect and quantify fluctuations in brain estrogens *in vivo* provides a direct means to explore how brain estrogen production contributes to both differentiation and neuromodulation during development. Here, we use this method to test the hypothesis that neuroestrogens are sexually-differentiated as well as dynamically responsive to song tutoring (via passive video/audio playback) during the period of song learning in juvenile zebra finches. Our results show that baseline neuroestradiol levels in the caudal forebrain do not differ between males and females during an early critical masculinization window. Instead, we observe a prominent difference between males and females in baseline neuroestradiol that emerges during the subadult stage as animals approach sexual maturity. Second, we observe that fluctuating neuroestradiol levels during periods of passive song tutoring exhibit a markedly different profile in juveniles as compared to adults. Specifically, neuroestrogens in the caudal forebrain are elevated following (rather than during) tutor song exposure in both juvenile males and females, suggesting an important role for the early consolidation of tutor song memories. These results further reveal a circadian influence on the fluctuations in local neuroestrogens during sensory/cognitive tasks. Taken together, these findings uncover several unexpected features of brain estrogen synthesis in juvenile animals that may have implications for secondary masculinization as well as the consolidation of recent sensory experiences.

Keywords

sex differences; song system; neuromodulation; cortex; memory; nongenomic

INTRODUCTION

Estrogens can act as powerful masculinizing agents early in development, often via the local conversion of circulating androgens via brain aromatase (Simpson and Vicario, 1991; Adkins-Regan et al., 1994; MacLusky et al., 1994; Hutchison, 1997). Estrogens are also known to influence a variety of vital brain functions such as memory, sensory processing, and cognition in both males and females (McEwen, 2002; Gulinello et al., 2006; Barha and

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Galea, 2010; Zimmerman et al., 2011; Banerjee and Liu, 2013). This diversity of actions of estrogens can be partially explained by multiple receptor subtypes expressed in divergent brain regions (Greco et al., 2001; Gottsch et al., 2009; Srivastava and Evans, 2013). In addition, estrogens can act on brain circuits on a variety of timescales that range from days-to-weeks (e.g., sexual differentiation) to seconds-to-minutes (e.g., neuromodulatory actions; Woolley, 2007; Vasudevan and Pfaff, 2008). To help clarify how estrogens shape and modulate brain circuits during development (e.g., Konkle and McCarthy, 2011), it therefore has become important to directly measure how estrogens change dynamically within discrete brain areas.

Brain steroids such as estrogens can now be monitored *in vivo* using microdialysis (Remage-Healey et al., 2008; Kenealy et al., 2013; Ubuka et al., 2014). Experiments using this method have focused on the auditory cortex of adult zebra finches (Remage-Healey et al., 2008; 2012), in which aromatase expression and activity is enriched (Vockel et al., 1990; Schlinger and Arnold, 1992; Schlinger and Arnold, 1993; Saldanha et al., 2000; Saldanha et al., 2011). One auditory cortical region, the caudomedial nidopallium (NCM; similar to secondary auditory cortex in mammals) provides the major source for local levels of estrogens within the broader ‘song system’ circuitry that is vital to audition and song production (Remage-Healey, 2012). The NCM is also considered a focal point for the storage of tutor song representations and long-term memories (Phan et al., 2006; Terleph et al., 2007; London and Clayton, 2008; Moorman et al., 2011; Yoder et al., 2012), in concert with other regions of the songbird brain (Prather et al., 2010; Roberts et al., 2012). Within the NCM, local estradiol levels are elevated during brief (~30 min) social interactions and in response to song playback. Local elevations in estradiol therefore occur during auditory activation of the NCM, in both male and female adult zebra finches (Remage-Healey et al., 2008; 2012). Elevations in local estradiol levels appear to have functional consequences for brain function, as estrogens can acutely enhance the auditory response-properties of neurons within NCM itself (Tremere et al., 2009; Remage-Healey et al., 2010; Tremere and Pinaud, 2011; Remage-Healey, 2012). These rapid actions of estradiol in NCM also enhance auditory representations in downstream regions of the song system, including sensorimotor cortex (Remage-Healey and Joshi, 2012). Thus, acute neuroestrogen fluctuations have consequences for auditory-dependent behaviors; disrupting NCM estrogen production leads to short-term deficits in song preference behaviors (Remage-Healey et al., 2010; Tremere and Pinaud, 2011). Together, this work has led to the conclusion that brain-derived estrogens are key modulators of neural circuit function and behavior in the adult forebrain. By contrast, it is less clear whether dynamic fluctuations in brain estrogen levels modulate brain function during development.

The current study examines both short-term and long-term patterns in brain estrogen concentrations during the song learning period in juvenile male and female zebra finches. Song learning in juvenile songbirds has received considerable attention to help in understanding the neurobiology of critical periods, behavioral plasticity, and sex differences (Adkins-Regan et al., 1994; Jarvis et al., 1995; Arnold, 1997; Gong et al., 1999; Brainard and Doupe, 2002; Konishi, 2004; Mooney, 2009). We focus here on song learning to provide clues about how brain estrogen production may impact learning and memory. In

many animal species, including humans, exogenous estrogen treatments can promote performance on learning and memory tasks (Luine et al., 1998; Luine et al., 2003; Bartholomeusz et al., 2008; Sherwin and Grigorova, 2011; Frick, 2012; Zhao et al., 2012; Bailey et al., 2013; Rensel et al., 2013). There are also growing indications that estrogen synthesis can itself drive enhanced learning and memory within specific brain regions, particularly in the hippocampus (Frick, 2012; Bailey et al., 2013). In this exploratory study, we examine the temporal dynamics of neuroestrogens in sensory cortex during a developmental time when young songbirds are acquiring and consolidating early vocal memories. Importantly, both male and female zebra finches must acquire memories for songs early in development that are used later in adulthood to guide song production (males only) and auditory discrimination and preference behaviors (males and females, see Holveck and Riebel, 2007).

A second goal of this study is to test whether divergent levels of *in vivo* brain estrogens emerge during development in male vs. female zebra finches. A role for brain-derived estrogens in the developmental masculinization of the zebra finch forebrain has been suggested by three primary lines of evidence. First, the enzymes necessary for the synthesis of 17-beta-estradiol (E2) *de novo* from cholesterol are expressed in the brain of juvenile zebra finches from hatching onward (Shen et al., 1995; Jacobs et al., 1999; London et al., 2006; London and Schlinger, 2007; London et al., 2010; London, 2013). Second, although female zebra finches ordinarily do not sing, treatment with exogenous E2 within the first two weeks after hatching can potently masculinize the brain, resulting in females that are able to sing in adulthood (Gurney and Konishi, 1980; Nordeen et al., 1986; Konishi and Akutagawa, 1988; Adkins-Regan et al., 1994; Grisham et al., 2008; Thompson et al., 2011). Third, *in vitro* culture studies have shown that local E2 production is both necessary and sufficient for masculinizing the synaptic connections between two prominent song nuclei at ~ 25-30 days (Holloway and Clayton, 2001).

Despite these indications, circulating levels of estrogens are largely undifferentiated in male and female zebra finches across the critical ‘masculinization window’ age range between 25-45 days (Hutchison et al., 1984; Adkins-Regan et al., 1990; Schlinger and Arnold, 1992). Together, these observations drove our interest in further testing whether sex differences in neuroestrogen levels within the brain are detectable *in vivo* during juvenile development in zebra finches.

MATERIALS AND METHODS

Rearing

All animals were raised from the University of Massachusetts, Amherst, breeding colony (14/10 h light/dark cycle). Between 14-19 days post hatching (dph), nests of hatchlings were removed from the main aviary room and placed in individual sound attenuation chambers (Eckel Noise Control Technologies) with their mother or an adult female care provider. Since female zebra finches do not sing, this rearing environment ensured that the juveniles would be naïve to any song stimulus at the onset of their sensitive period, prior to experimental playback exposure (Eales, 1985; 1987). Hatchlings were sexed genetically (see below) and once they were able to feed themselves (~ 20 dph), all animals were relocated

into cages in separate sound attenuation chambers. Juvenile males were separated from each other and females were separated from males as a means to prevent each focal subject from hearing juvenile male subsong other than its own. Each juvenile was provided with at least one adult female companion. EMW omnidirectional lavalier microphones (Countryman Associates) were placed in all chambers, and songs were automatically recorded throughout using Sound Analysis Pro. A subset of experimental males were reared in the breeding colony until 30-40 dph. These individuals were therefore exposed to song prior to tutor playback ($n = 8$). No significant differences in neuroestrogen baseline levels or neuroestrogen levels in response to tutor playback were observed between naïve and exposed juvenile males. All females in this study were naïve to song. The sample sizes in this study for juveniles reared and completed through microdialysis testing were ($n = 32$ males and $n = 14$ females). To maximize our experimental yield, over the course of 3-4 days of microdialysis individual animals were exposed to more than one treatment (treatments described below) on successive days. No two treatments were administered on the same day per animal to avoid carry-over effects. For example, an individual male was exposed to tutor song on day 1, followed by ‘audio only’ on day 2, and pharmacological treatment on day 3, and the order of these treatments was counter-balanced across animals whenever possible.

Sex determination

Since the critical period for sensory learning occurs prior to the emergence of adult plumage, the sex of hatchlings was determined by PCR. Whole blood was collected from the brachial vein and purified for DNA with QIAamp DNA mini kits (QIAGEN, Cat. no. 51306). Genomic PCR amplified fragments of the CHD (chromohelicase-DNA-binding) genes found on avian sex chromosomes, using a pair of degenerate primers (P2: YTKCCAAGRATGAGAACTG and P8: TCTGCATCACTAAKCCTTT), as previously described (Griffiths et al., 1998). Sex was then determined via electrophoresis on 2% agarose gel, with males (ZZ) presenting one distinct fragment and females (ZW) presenting an additional band differing by 36 bp.

Cannula surgery

Surgical implantation of a microdialysis guide cannula (adapted from Remage-Healey et al., 2008; 2010; 2012) was performed 4-5 days prior to *in vivo* microdialysis, to ensure full recovery prior to experiments. For all surgeries, animals underwent general anesthesia via intramuscular injection (Equithesin, 3.2 ml per kg of body weight) and were maintained in a custom stereotaxic device (head angle, 45°; Herb Adams Engineering) at 37°C with a heating pad (FHC Neurocraft) for the duration of the procedure. Birds first received a subcutaneous injection of local anesthetic (lidocaine, 2% in ethanol) under the scalp. Using stereotaxic coordinates, a small incision was made to expose the skull dorsal to NCM. A microfenestra was made through the outer- and inner-leaflet of skull with a 26 G needle tip, and the dura mater was carefully resected to allow cannula penetration. A CMA-7 microdialysis guide cannula with obturator (CMA Microdialysis, CMA 7, Ref. No. P000138) was implanted into left NCM (1.2 mm anterior, and 0.9 mm lateral of the mid-sagittal sinus, 1.0 mm below surface of the dura mater) through this fenestra. Histological inspection of an initial set of experiments showed that coordinates for implantation into NCM needed to be adjusted for the juvenile nidopallium. Results from these experiments are

presented below as a group of ‘lateral nidopallium’ experiments. This group provides an important comparison set of results of baseline and fluctuating estradiol levels for ‘Microdialysis misses’ (see Results below) distinct from experiments with probes directed successfully directed at NCM. The guide cannula was secured with cyanoacrylate and dental cement (Perm Reline/Repair Resin; Coltène/Whaledent), and the incision site was sealed with cyanoacrylate. Following surgery, all birds were returned to their separate cage alongside a companion adult female (total duration of removal < 90 min).

***In vivo* microdialysis**

In preparation for *in vivo* microdialysis, the cannula obturator was replaced with a CMA/7 MD probe (1mm membrane length, CMA Microdialysis, Ref. No. P000082) prefilled with artificial cerebrospinal fluid (aCSF) at 2 µl/min as in previous studies (Remage-Healey et al., 2008; 2010; 2012). Birds were housed individually (no other birds present) inside in a custom cage inside sound attenuation chambers (Eckel Industries), with FEP inlet and outlet tubing connected to a low-torque quartz-lined dual-channel microdialysis swivel (375/D/22QM; Instech Labs). Tutor playback experiments began the following day, at least 15 hours following implantation. All birds were confirmed to be active and engaged in normal feeding, drinking, flying and hopping behaviors prior to the start of experiments.

Pharmacological inhibition of aromatase

To test the validity of our *in vivo* approach for measuring neuroestrogens in juvenile brain, we administered the aromatase inhibitor fadrozole (FAD; 100 µm) via reverse-microdialysis (retrodialysis) to a subset of male subjects. These experiments were carried out in three males during the last day of their respective active microdialysis sampling period on day 4 (i.e., at the completion of tutoring experiments and prior to sacrifice). All three subjects had probes confirmed to be within NCM (see histological analysis below). In adults, FAD retrodialysis is known to cause a rapid suppression of local estradiol levels as measured by microdialysis (Remage-Healey et al., 2008). In males of ages 38, 44, and 62 dph the same dose of FAD was administered for 60 min after two 60 min periods of aCSF ‘pre’ periods to establish baseline E2 levels. Two 60 min periods of aCSF ‘post’ periods followed the FAD treatment to assess washout conditions.

Playback of tutor song

Prior studies have shown that in adult males and females, local E2 levels in NCM are elevated during acute periods of song playback (Remage-Healey et al., 2008; 2012). Although adult females do not sing, in both males and females song preferences are shaped early on by tutor exposure (e.g., Holveck and Riebel, 2007). We therefore tested whether local estradiol levels within NCM in juvenile males and females respond to auditory and visual stimulus of an adult male tutor. We collected dialysate before, during, and after presentation of video tutor playback. Video presentations were used to provide an identical audiovisual stimulus for all birds in the study. Dialysate samples were collected during the photophase, hourly from 8AM to 6PM. Tutor playback trials consisted of a morning trial from 10-11AM, and an afternoon trial from 3-4PM. Subjects were exposed to tutor playback via a USB LCD monitor (Lilliput 7” TFT LCD) and stereo minispeakers (Sony, model#

SRS-TP1WHI). All animals received the same tutor playback, which consisted of an hour-long video of a singing adult male coupled to audio with amplitude peaks at ~ 70dB. The screen was placed at ~ 8 cm from the cage wall and the video of the adult male was adjusted to be proportionate to a life-size male (songs in the video were directed at a female placed behind the camera). The video/audio setup inside the microdialysis chamber was very similar to a previous video/audio playback study with adult males and females (Ramage-Healey et al., 2012). The video was composed of three 3-minute video clips of the same singing adult male, interspersed with 5 minutes of baseline stimuli between clips (black screen, no sound), and looped for 60 minutes.

Microdialyzed birds received 2 hours of no auditory or visual playback (silence and blank screen) prior to and after tutor playback, during which we recorded their vocalizations for song (males only) and general activity levels. Dialysate samples were therefore collected in 60 min bins to allow them to be run in duplicate on ELISA (2× 50 µl samples). Tutor playbacks were videotaped and analyzed to confirm that birds were engaged with the video/audio playback. A subset of birds were exposed to control treatments during similar trials, consisting of either a blank screen + tutor song audio ('audio only'), a video of a tutor with no audio ('video only') and results for 'video only' (n = 6 males & n = 11 females) and 'audio only' (n = 8 males) treatments are presented below (see 'Visual vs. auditory tutoring'). Dialysate was stored immediately after collection at -80 °C.

To examine potential sex differences during development, a set of baseline E2 levels were collected from NCM prior to each day's tutor playback experiment. Raw baseline E2 levels were averaged per bird over at least 3 days and these average E2 levels were analyzed for between-subject differences for the variables sex (male vs. female) and age (average dph).

To allow comparisons of neuroestrogen levels fluctuating during and after song presentation between juveniles and adults, a separate set of N = 11 adult males (> 140 dph) underwent microdialysis with probes targeted to NCM. These males were exposed to audio and video playback of tutor songs in the morning, as outlined above for juveniles (60 min bins). Results from these adult males are plotted in Fig. 6 for comparison with juvenile males.

Enzyme-Immunoassay

A commercial ELISA kit (Cayman Chemical 582251) was used to measure E2 levels in all collected dialysate samples, following established protocols (Ramage-Healey et al., 2008; 2012). All samples were run in duplicate (each original ~ 120 µl sample was split into two 50 µl samples for analysis). In addition to dialysate, for each assay plate we ran several samples of the same aCSF batch (i.e., identical aCSF solution that was infused during a given bird's experiment) to determine the 'background' concentration of E2 as reported by the assay. The background E2 level in aCSF alone (e.g., not perfused through the microdialysis system) was on average 4.47 ± 0.69 pg/ml for aCSF for the entire study. Intra-assay CV of duplicate samples within each 96-well plate was 10.52%, while inter-assay CV of standard spiked samples across plates was 21.42%. To standardize comparisons among raw E2 levels across ages, sexes, and ELISA runs, the background aCSF level of E2 as reported by each assay was subtracted from dialysate E2 levels from NCM for that assay run. Therefore all absolute levels are reported below as 'baseline E2', and they reflect our

estimate of detectable concentrations of estradiol in the auditory forebrain. All samples from each animal were assayed in the same ELISA plate to diminish the contribution of inter-assay variation to variability in our estimate of neuroestradiol fluctuations for the tutor playback experiments.

Song recording and analysis

In a subset of experiments, males were confirmed singing prior to surgery, (ages 44-60 dph). This allowed for song measurements from males pre-vs. post-tutoring. All songs were recorded within anechoic chambers (Eckel), using EMW omnidirectional lavalier microphones (Countryman Associates Inc.), connected to a PCA2 Stereo Power amplifier, AudioBox USB audio mixer, Enhanced 8 channel PreAmp, and FP10 Firewire Recording Interface. Songs were then sorted and analyzed via Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu/sound_analysis_pro). Two song features were the focus of analysis in young juvenile males, Wiener entropy and Wiener entropy variance. These two features have previously been documented to reflect changes in song that occur following tutoring during the sensorimotor stage (Deregnaucourt et al., 2005; Shank and Margoliash, 2009). An upward shift in entropy variance is thought to reflect the incorporation of new song elements and variability into song following initial tutor exposure.

Histological analysis

Following the conclusion of tutor playback experiments all birds were subsequently sacrificed via anesthetic overdose and perfusion to determine probe/cannula placement. Brains were dissected and sunk in a 20% sucrose/formalin solution for 12 hours and then frozen at -80°C in embedding molds with TissueTek OCT. Brains were sectioned parasagittally at 45 μm on a cryostat and immediately thaw-mounted onto slides (Fisher superfrost). Following a 24-hour drying period in -4°C , slides were dehydrated, stained with thionin, and coverslipped. Slides were analyzed and photographed using a light microscope (Zeiss) to identify probe placement. Slide images presented in Fig. 2 are from whole-brain scans (Brother) to provide representative probe placements in the medial-lateral extent of the sagittal plane.

Statistics

All results were analyzed using either Statview 4.5 (for repeated-measures ANOVAs) or JMP (for regression analyses) with significance set to $p < 0.05$. Whenever possible in a balanced design, multi-way repeated measures ANOVA were run to include time-of-day, sex, and any independent treatment variable in the model. For comparisons between sexes of baseline E2 levels, unpaired T-tests were run when the overall model was significant for the variable 'sex'. For comparisons across time of either hormone levels or song parameters, Wilcoxon signed-rank post hoc tests were run when the overall model was significant. Wilcoxon paired tests are robust to low sample sizes and do not assume parametric distribution of dependent variables.

RESULTS

Pharmacological inhibition of aromatase

Our initial experiments set out to test the validity of our *in vivo* approach for measuring endogenous fluctuating neuroestrogens in juvenile zebra finches (for similar experiments in adults see Remage-Healey et al., 2008; 2012). We administered the aromatase inhibitor fadrozole (FAD; 100 μ m) via reverse-microdialysis (retrodialysis) to three juvenile male subjects with probes confirmed to be targeted to NCM. Prior work in adults has shown that FAD retrodialysis causes an acute suppression of local estradiol levels (Remage-Healey et al., 2008). Similarly, for N = 3 males of ages 38, 44, and 62 dph the same dose of FAD caused a suppression of local estradiol levels in NCM (Fig. 1; repeated-measures ANOVA for the first three periods $F_{(2,4)} = 10.450$; $p = 0.026$). Therefore, pharmacological inhibition of estrogen synthesis in NCM caused a direct suppression of estradiol levels as measured by our microdialysis method in juveniles. Taken together with the results of previous work, this experiment confirmed the validity of microdialysis to measure endogenous fluctuations in neuroestrogens in juveniles.

Microdialysis ‘misses’ reveal the region-specificity of juvenile E2 fluctuations

Inspection of histological sections from our experimental subjects showed that for five male juveniles (ages 37,44,63,78 and 80 dph) microdialysis probes were sampling from the lateral nidopallium, i.e. at least 1.0 mm lateral from the caudomedial nidopallium. This mistaken probe placement was due to an early miscalculation in stereotaxic coordinates for NCM in juveniles. Representative sections are shown in Fig. 2 for probe placements within NCM vs. within the lateral nidopallium. We pooled results from the five procedural ‘misses’ in the lateral nidopallium together as a comparison group for the results presented below for probes targeted correctly within NCM. We present here results from the lateral nidopallium group to examine the region-specificity for baseline levels of E2 as well as tutoring-dependent changes in E2 within the NCM.

As shown in Table 1, baseline estradiol levels for *in vivo* microdialysis in juveniles in lateral nidopallium (‘misses’) were significantly lower than baseline values from samples collected within NCM in age-matched juveniles. This is consistent with the distribution of the estrogen-synthesis enzyme aromatase in the nidopallium in zebra finches, which is elevated in the medial nidopallium as compared to the lateral nidopallium (Shen et al., 1995; Saldanha et al., 2000; London et al., 2006; London and Schlinger, 2007). Therefore, the lower concentrations of estradiol observed in the lateral nidopallium as compared to NCM are in agreement with the probe placement in a brain region largely lacking constitutive aromatase (for further discussion of region-specificity, see also Schlinger et al., 2014). Implantation of microdialysis can induce an up-regulation of reactive aromatase in glia surrounding the implantation site (Remage-Healey et al., 2008), similar to other perturbations in the zebra finch brain (Peterson et al., 2004; Duncan et al., 2012). This reactive glial response could therefore cause interference with our measurements of estrogens using microdialysis. However, since we can expect the reactive glial aromatase response to be similar in the lateral and medial nidopallium (for similar ideas regarding the entopallium, see Duncan and Saldanha, 2011) the lower concentrations of estradiol observed

in the lateral nidopallium observed here also are consistent with the hypothesis that constitutive neuronal (and not reactive glial) aromatase contributes a large portion of the detected estradiol concentrations using this method.

Similarly, as shown in Table 2, in contrast to our findings in NCM (see below) the levels of estradiol in the lateral nidopallium were essentially unresponsive to our tutoring paradigm. The levels of E2 were low and did not shift during or following playback in the lateral nidopallium in either the morning or afternoon session for $N = 5$ males. Multi-way repeated-measures ANOVA revealed no effect of time-of-day ($F_{(1,32)} = 0.076$; $p = 0.79$), no effect of tutor playback ($F_{(4,32)} = 0.678$; $p = 0.61$), and no interaction between tutoring*time-of-day ($F_{(4,32)} = 0.80$; $p = 0.53$) on estradiol levels in the lateral nidopallium.

For experiments in NCM, we standardized E2 levels to baseline values to account for age-dependent changes for probes targeted correctly within NCM (see below). To allow direct comparisons with results from our lateral ‘misses’, all microdialysis E2 levels from the lateral nidopallium group were also normalized to 100% of the baseline for each bird and then re-tested for the effects of time-of-day and tutoring. Similar to non-normalized data, multi-way repeated-measures ANOVA revealed no effect of time-of-day ($F_{(1,24)} = 0.10$; $p = 0.75$), no effect of tutor playback ($F_{(3,24)} = 0.506$; $p = 0.68$), and no interaction between tutoring*time-of-day ($F_{(3,24)} = 0.21$; $p = 0.89$).

Together, these results from dialysate collected from the lateral nidopallium stand in stark contrast with the patterns observed below for neuroestrogen levels collected within NCM. All of the remaining experiments described below are from animals with probe placements histologically confirmed to be sampling within NCM.

Effects of age and sex on estradiol in NCM

Baseline E2 levels in NCM for all juvenile males and females in this study were compared for the effects of age and sex. We treated average age (e.g., mean age of each bird over the ~ 5 experimental days of microdialysis) as a variable in two ways for analysis. Average age for each bird was analyzed as a binned discontinuous variable (i.e., age bins were ‘sensory’ (25 – 35 dph), ‘sensorimotor’ (36 – 45 dph), and ‘subadult’ (46 – 80 dph) for the purpose of multi-way ANOVA. In a separate analysis, average age was treated as a continuous variable for the purposes of linear regression. The age bins correspond approximately to the species-typical developmental transitions from an early critical period of sensory acquisition of tutor song that is prior to subsong (25-35 dph), an active period of male sensorimotor refinement of learned songs (30-60 dph), and a period of subadulthood prior to the completion of sexual maturity in both sexes (60-80 dph; see Bailey and Wade, 2005; Adkins-Regan and Leung, 2006; Mooney, 2009)

Multi-way ANOVA revealed a significant effect of sex ($F_{(1,33)} = 4.690$; $p = 0.038$), a significant effect of post-hatching age ($F_{(2,33)} = 7.404$; $p = 0.022$), and a significant interaction between sex*post-hatching age ($F_{(1,50)} = 3.783$; $p = 0.033$) on baseline E2 levels in NCM. As shown in Fig. 3, T-tests revealed that no significant sex differences in baseline E2 levels occurred at either the sensory age ($T_{(8)} = -0.447$; $p = 0.666$) or the sensorimotor age ($T_{(14)} = -0.144$; $p = 0.888$). However, by the subadult age, males had significantly

higher baseline E2 levels in NCM ($T_{(11)} = -3.784$; $p = 0.003$). No effects of time-of-day were observed for baseline E2 levels for either males ($p > 0.48$) or females ($p > 0.56$).

Linear regression analyses of baseline E2 data treated as a continuous variable supported the same overall conclusion (Fig. 4). That is, NCM E2 levels became elevated in males as compared to females as they reached the subadult age range (after 50 dph). In males, there was a significant, linear effect of age on NCM E2 levels, with age treated as a continuous variable in linear regression ($F_{(1,23)} = 30.718$; $p < 0.0001$; $R^2 = 0.57$). By contrast, in females there was no significant effect of age, treated as a continuous variable in linear regression ($F_{(1,23)} = 1.349$; $p = 0.268$; $R^2 = 0.10$). Taken together, both ANOVA and linear regression analyses showed that a prominent sex difference for *in vivo* neuroestrogen levels emerged late in development, prior to sexual maturity.

Effects of tutoring and time of day on estradiol in NCM

Since our results with raw baseline E2 levels in NCM showed significant variation by sex and by age, we standardized all measures of fluctuating E2 levels to allow comparisons of the response to tutoring across ages and sexes. To accomplish this standardization, we normalized all microdialysis E2 levels for the tutor playback study to 100% of the average E2 level during the pre-tutoring period for each bird. In other words, for each individual we normalized all NCM E2 levels for any trial to the baseline 'pre' NCM E2 levels for that trial.

Initial inspection of our results showed an apparent effect of the time-of-day on the response of local E2 levels in NCM to tutoring (see Fig. 5). A multi-way repeated measures ANOVA showed a significant effect of time-of-day on E2 levels across all periods (i.e., morning vs. afternoon; $F_{1,50} = 5.442$; $p = 0.024$), as well as a significant effect for a time-of-day*tutoring interaction ($F_{1,234} = 2.049$; $p = 0.025$) but no age*tutoring interaction ($p > 0.43$). These initial results revealed that presenting tutor playbacks in the morning vs. the afternoon elicited different patterns for changing E2 levels in the NCM of juveniles. They also indicated that changing E2 levels occurred similarly in animals of all age groups. Because an overall trivariate model was unbalanced with respect to sex*tutoring*time-of-day, we elected to analyze responses to tutoring in the morning vs. afternoon separately, as outlined below.

For tutor playback experiments conducted in the morning, repeated measures ANOVA revealed a significant effect of tutoring ($F_{3,114} = 3.594$; $p = 0.016$), and no effect of sex ($F_{1,38} = 0.202$; $p = 0.655$) or a sex*tutoring interaction ($F_{3,114} = 0.224$; $p = 0.879$). As shown in Fig. 5A, Wilcoxon post hoc tests revealed that NCM E2 levels decreased during the tutor playback period for males and females ($Z = -2.083$; $p = 0.037$) and increased in the first 'post tutor playback' period for males and females (post 1 vs. tutor; $Z = 2.729$; $p = 0.006$), and then returned to baseline (post 2 vs. post 1 $Z = -2.511$; $p = 0.011$). Therefore, for both male and female juveniles, NCM E2 levels were suppressed during tutoring itself but levels immediately rose during the first post-tutoring 60-min period.

By contrast, for tutor playback experiments conducted in the afternoon, repeated measures ANOVA revealed no effects of tutoring ($F_{3,99} = 0.565$; $p = 0.639$), sex ($F_{1,33} = 1.071$; $p = 0.308$), or a sex*tutoring interaction ($F_{3,99} = 0.577$; $p = 0.631$). As shown in Fig. 5B, NCM

E2 levels were unchanging during or following tutoring in experiments conducted in the afternoon.

Taken together, these results reveal a previously undocumented effect of time-of-day on the ability of the brain to rapidly modulate local neuroestrogens during sensory/cognitive tasks. In addition, in comparison with adults in which NCM E2 levels are elevated during song presentation, juvenile zebra finches exposed to song tutoring exhibit an elevation in NCM E2 levels only during the silent period following the playback, after the song presentation has ceased (see Fig. 6 for comparison between adult and juvenile males for song exposures in the morning).

Effects of tutoring on song parameters

A subset of 8 male zebra finches (ages 44-60 dph) were analyzed for changes in song parameters as a result of our 5-day paired video-audio tutoring paradigm. These males were chosen because they reliably sang 'plastic' song in the days leading up to the initial tutoring session, which allowed comparisons of song parameters before and after the onset of tutoring. Ten renditions of song were selected from each male's songs for each day of the experiment, and song parameters were averaged for each day. Repeated measures ANOVA revealed a significant effect of tutoring on both daily average Wiener entropy variance (Fig. 7; $F_{4,28} = 3.653$; $p = 0.016$), as well as daily average Wiener entropy (data not shown; $F_{4,28} = 7.141$; $p = 0.0004$). Wilcoxon post hoc tests revealed that entropy variance was significantly elevated on the last day of tutoring (last vs. pre; $Z = -2.751$; $p = 0.028$). Thus, song parameters for microdialyzed males were significantly affected by the video tutor playback regime, although we did not observe mean changes in Wiener entropy variance that were of the magnitude reported by others using live or operant tutoring paradigms (e.g., Deregnaucourt et al., 2005). A separate set of male zebra finches who were not part of this study but were exposed to a similar passive video tutor playback regimen were evaluated for tutor song similarity in adulthood (the birds in the current study were all sacrificed prior to adulthood to determine probe placement). This analysis showed that passive video tutor playback (i.e., not operantly-elicited) produced song similarity scores in the range of 35-60%, which is well below the song similarity scores ~80% achieved by live tutoring (D. Vahaba and L. Ramage-Healey, unpubl obs) or operant tutoring (Deregnaucourt et al., 2005; 2013). In sum, our passive video/audio tutoring playback paradigm produced modest changes in vocal parameters in males undergoing *in vivo* microdialysis.

Unimodal visual and auditory tutoring

A subset of 6 males and 11 females were exposed to audio tutoring alone ('audio only') during microdialysis trials in the morning. The overall ANOVA revealed that there was no effect of 'audio only' tutoring (Fig. 8; $F_{3,45} = 0.261$; $p = 0.853$) no effect of sex ($F_{1,45} = 0.741$; $p = 0.403$), and no sex*audio tutoring interaction ($F_{3,45} = 0.342$; $p = 0.795$) on NCM E2 levels. Similarly, for a subset of 8 males that were exposed to tutor video in silence ('video only'), there was no effect on NCM E2 levels (data not illustrated; $F_{3,21} = 1.891$; $p = 0.162$). Therefore, these experiments indicate that the combined sensory modalities of visual and auditory song tutoring together were associated with fluctuations in NCM E2 levels in juvenile males and females.

DISCUSSION

Several results in this study were novel and largely unexpected. First, contrary to our prediction, we did not detect a sex difference in baseline E2 levels in the caudal forebrain of juvenile zebra finches during the critical masculinization time period. Second, fluctuating E2 levels in response to tutoring exhibited a distinctly different profile in juveniles as compared to adults. Third, these results revealed a previously undocumented effect of time-of-day on the ability of the brain to rapidly modulate local neuroestrogens during sensory/cognitive tasks. These three main themes are discussed below.

***In vivo* brain estrogens do not account for early masculinization**

Our findings regarding baseline E2 levels in NCM are consistent with the hypothesis that E2 levels produced locally in the brain of developing zebra finches are associated with the emergence of a masculinized brain. However, in contrast to previous observations *in vitro* (Holloway and Clayton, 2001) the current work does not support the hypothesis that estrogen production in the caudal forebrain (of which NCM is the primary source) can account for an early masculinization period at post-hatch day 25-40. Indeed, we did not detect a significant difference in baseline caudal forebrain E2 levels between males and females during the critical ages of sensory and sensorimotor development. A large sex difference did, however, emerge for caudal forebrain E2 levels during the subadult stage (> 45 dph). Therefore, prior to sexual maturation, baseline E2 levels are substantially higher in the caudal forebrain of male vs. female zebra finches.

It remains likely that other steroid microenvironments outside the NCM could account for the masculinization at ~ 25dph. One candidate for this masculinization by brain-synthesized estrogens is HVC, a critical sensorimotor nucleus that contains sex-specific aromatase fibers (Saldanha et al., 2000). A second candidate is the region immediately surrounding RA (Jacobs et al., 1999). It is likely our microdialysis method is unable to detect such neuroestrogen microenvironments in the caudal forebrain (such as those within HVC) with sufficient spatial resolution. At the same time, however, it is clear that our analyses emphasize elevated neuroestrogen levels and fluctuations that occur in the NCM and not the lateral nidopallium, consistent with a degree of region-specificity as was previously reported in adults (Remage-Healey et al 2008; 2012).

If the sex difference in baseline E2 levels that emerges in the late juvenile/subadult stage is involved in the emergence and/or maintenance of masculinized song circuitry, the rising neuroestrogen tone in the brain of males is undoubtedly acting in concert with other hormonal and neurogenetic factors during this developmental timeline (Jin and Clayton, 1997; London et al., 2009; Tomaszewski et al., 2009; London, 2013). There is abundant evidence that E2 acts during a developmental time period (i.e., within the first two weeks) to masculinize the song system, and the masculinizing effects of E2 on select song system nuclei can still be observed up to ~ 45 dph (Konishi and Akutagawa, 1988). After this point in development, neuronal apoptosis in the song system in females appears to preclude any further masculinizing effects of E2. It is interesting to note, therefore, that we observed the emergence of the sex difference in baseline E2 levels in the present study in the 'subadult' group (> 46 dph), only after they have passed this 45-dph masculinization threshold. By this

reasoning, the sex difference in baseline E2 levels uncovered here using microdialysis in the caudal forebrain does not account for the first-order masculinization of the song circuitry. We reason that estrogens are involved in late maturation or maintenance of the male zebra finch caudal forebrain. Prior investigations have been unable to detect a substantial sex difference in juvenile or adult zebra finches in circulating estradiol concentrations (Adkins-Regan et al., 1990) as well as in local estradiol levels in NCM (Remage-Healey et al., 2012). Therefore, the elevation in baseline caudal forebrain estrogens in males may be a transient event during late sexual maturity. The emergence of elevated levels of estrogens in NCM in subadult males could reflect the demands of auditory-motor integration and plasticity that is occurring at this age, prior to song crystallization at ~90-100 dph.

Now that the annotated zebra finch genome has become available (Warren et al., 2010), it is possible to examine hormone-gene interactions by probing for estrogen-response elements and/or transcripts in response to divergent neuroestrogen actions in the male vs. female brain. Our findings that neuroestrogen levels within the caudal forebrain are undifferentiated during the critical period in which exogenous E2 masculinizes the brain further emphasize the need to examine other contributors to the E2 signaling pathway that may account for the masculinization. Promising candidates include steroid co-activators, nonclassical receptors, and second-messengers (Duncan and Carruth, 2007; Thompson et al., 2011; Acharya and Veney, 2012; Adkins-Regan, 2012; Tang and Wade, 2012; Wade et al., 2013).

The sex difference in baseline E2 levels that emerges late in the juvenile period has several potential proximate explanations. First, the activity of aromatase in the caudal telencephalon could be elevated in males as compared to females at this stage (e.g., Peterson et al., 2005; Rohmann et al., 2007). Second, brain aromatase activity may not differ between sexes, but the substrate availability for aromatization itself may be changing. One prediction deriving from this hypothesis is that elevated androgens may be more bioavailable in the NCM of males as compared to females as gonadal androgen secretion ramps up during the approach to sexual maturity (Schlinger and Brenowitz, 2008). Third, the activity of other enzymes or proteins that may convert, conjugate, or sequester available E2 may be more active in females than in males during the subadult stage. In this light, it is interesting to note that sex differences in the expression/activity of the key HSD17B4 enzyme have been reported in some brain regions in this species during development (Tomaszycki et al., 2009; London et al., 2010; Thompson et al., 2011).

***In vivo* brain estrogens fluctuate in response to tutoring**

The second principal finding of this study is that the exposure to passive video/audio playback of tutor songs (in the morning but not afternoon) caused an elevation in NCM E2 levels in both males and females within the first 60 min after tutoring. Our method of song tutoring via passive video/audio playback allowed precise standardization across individual juveniles undergoing microdialysis, but this method does not yield high copying fidelity in comparison with live tutoring. Thus, there may be critical differences in the social/perceptual requirements for successful juvenile tutoring that are not mimicked by our method. Despite this caveat, unlike previous observations in adult males and females, in which NCM E2 levels are elevated during brief periods of song exposure (Remage-Healey

et al., 2008; 2012), juveniles of all ages exhibited an elevation in NCM E2 levels only after a brief period of tutor song exposure concluded. One hypothesis consistent with these results is that neuroestrogens are elevated immediately after tutor song exposure to support the short-term consolidation of memories for song. The rise in neuroestrogens immediately after song tutoring may be proximately linked to the activation of downstream MAPK signaling pathways such as the induction of *egr1* (ZENK) that are important for song memorization in NCM (Jin and Clayton, 1997; London and Clayton, 2008). Our findings with NCM neuroestrogens are also consistent with emergent findings in other forms of learning and memory. Specifically, the memory-enhancing effects of exogenous estrogens are particularly pronounced during the post-training consolidation periods for both spatial and object memory tests in rodents (Luine et al., 1998; Luine et al., 2003; Phan et al., 2011; Zhao et al., 2012). This reasoning suggests the hypothesis that post-tutoring elevations in neuroestrogens are associated with enhanced consolidation for recent tutor song exposures in NCM.

Alternatively, it may be that the most salient difference between the pattern for juveniles vs. adults in this study is the lack of elevation in juvenile NCM during the 60-min period of active processing and encoding of song (i.e., during the period of tutor song exposure; see Fig. 6). In fact, there may be a degree of sex-specificity here, since we detected a significant decrease in NCM estradiol only in males during tutoring. As an alternative to the ‘consolidation’ hypothesis presented above, it is possible that estradiol levels in NCM are specifically downregulated during auditory processing by the juvenile brain. Since estradiol can induce a host of acute cell-signaling cascades in neurons (Woolley, 2007; Frick, 2012; Srivastava and Evans, 2013; Laredo et al., 2014) it is possible that E2 levels are reduced during juvenile song learning to dampen immediate neuroplasticity-related events during intensive periods when songs are being actively encoded. These two hypotheses establish predictions about the memory-interfering effects of estradiol during song tutoring (which would favor the ‘plasticity dampening’ hypothesis), vs. the memory-interfering effects of suppressing estradiol synthesis following song tutoring (which would favor the consolidation hypothesis). Testing these predictions has now become an active area of research interest.

The developmental trajectory for neuroestrogen fluctuations is an intriguing aspect of these findings, and one that may inform our understanding of how estrogens shape vocal learning in songbirds (Prove, 1983; Marler et al., 1988) and in human infants (Wermke et al., 2014). At some point after sexual maturity, it appears that male and female zebra finches ‘switch on’ the ability to respond to periods of song exposure with concurrent elevations in local levels of neuroestrogens. Specifically, elevations in NCM estradiol levels during song processing are observed in both adult males (Remage-Healey et al 2008) and females (Remage-Healey et al., 2012), but not in juveniles of either sex up to 80 dph (present study). The functional significance of rapid elevations in estradiol in NCM during song exposure is becoming more clear for adult zebra finches, since neuroestrogens acutely enhance the auditory response-properties of neurons within NCM in both males and females (Tremere et al., 2009; Remage-Healey et al., 2010; Tremere and Pinaud, 2011; Remage-Healey et al., 2012). By contrast, it is unclear what role neuroestrogen fluctuations play in the patterning of neurons in the NCM of juvenile males and females. To date, the only available data show

that the propagation of rapid neuroestrogen actions between NCM and the downstream sensorimotor HVC is also only observed in mature adult males and not in juvenile males (60-80 dph; Remage-Healey and Joshi, 2012). Together these observations indicate that neuroestrogen synthesis and action within the caudal forebrain undergo a maturation process in the late juvenile period. It is possible that this maturation is linked to the elevated gonadal androgens that permanently alter the response properties of song system nuclei during the transition to adulthood (White et al., 1999). How this maturation is coordinated with other extragonadal steroid synthesis pathways that are also developmentally emergent in songbirds (e.g., Schmidt and Soma, 2008; Shah et al., 2011) remains an intriguing area for future research.

Neuroestrogen fluctuations are sensitive to time-of-day

These results reveal a previously undocumented effect of time-of-day on the ability of the brain to rapidly modulate local neuroestrogens during sensory/cognitive tasks. First, we found that baseline E2 levels in NCM were not different in the morning vs. the afternoon. This is consistent with earlier reports that neuroestrogens in the songbird brain (as measured by microdialysis) do not appear to fluctuate significantly over a circadian period in adults (Remage-Healey et al., 2008; M. Ikeda and L. Remage-Healey, unpubl. obs.). However, this study also shows that the acute responses of neuroestrogens to sensory stimuli are different in the morning vs. the afternoon. This observation adds to a growing body of work indicating that brain steroid signaling can change over the course of the day to account for varying physiological and/or modulatory demands (Tsutsui et al., 2008). For example, the synthesis of the pregnane neurosteroid, 7- α -hydroxypregnenolone, varies according to a circadian pattern that is tied to the daily rhythm in melatonin secretion (Tsutsui et al., 2008; 2009). It remains to be seen the extent to which neuroestrogen signaling is associated with circadian factors such as melatonin (see Laredo et al., 2014).

In the current study, significant neuroestrogen fluctuations were observed in the juvenile brain following tutoring only in the morning, and not in the afternoon. The functional significance of this pattern remains unclear, but it may be related to the daily variation in singing and song learning that occurs in songbirds. In male zebra finches during the developmental song learning period, song features such as entropy variance change over the course of the day, reflecting an interaction between factors such as vocal practice, sleep, and circadian rhythms (Deregnaucourt et al., 2005; Crandall et al., 2007; Shank and Margoliash, 2009). It is possible, therefore, that neuroestrogen synthesis in the morning following tutoring provides a mechanism for the consolidation of songs during the active 'dawn song' period of the early photophase. The current observation that both males and females exhibit an elevation in NCM estrogens post-tutoring in the morning emphasizes that this pattern is likely linked to sensory/memory aspects of song learning more broadly and not just motor/practice learning (i.e. vocal matching) in males exclusively.

Acknowledgements

The authors thank Laura Bernal-Corzo, Maaya Ikeda, Mike Stefanelli, and David Jeon for technical support during the course of this study. The authors declare no conflict of interests for this work. The work was supported by NIH R00NS066179, NSF IOS1354906 and the University of Massachusetts.

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Figure 1.

Reverse-microdialysis (retrodialysis) of the estrogen-synthesis inhibitor fadrozole (FAD; 100 μ M) leads to an immediate, transient suppression of estradiol (E2) in the NCM of juvenile males. Each time period is 60 min and during 'Pre' and 'Post' time periods aCSF alone was retrodialyzed. All samples were collected in series to determine changes over time (i.e., 'Pre 1' is a sample collection 120-60 min prior to the FAD trial and 'Pre 2' is 60-0 min prior to the FAD trial). * $p < 0.05$

Figure 2.

Histological sections from representative animals in two regions of the nidopallium. A. Probes were targeted successfully in the caudo-medial nidopallium (NCM) in the majority of animals in this study. B. In a few cases ($n = 5$) probes were mistakenly targeted to the lateral nidopallium, outside the established boundaries of NCM. Arrows indicate the site of entry for microdialysis probes in each section. Note: the section in panel B is lateral relative to panel A (lateral in the sagittal plane), which is representative of the ‘misses’ in the lateral nidopallium. Gliosis surrounding the probe site is evident as a darker Nissl stain in the section in panel B.

Figure 3.

A prominent difference in baseline neuroestradiol levels emerges between males and females in a late juvenile stage prior to sexual maturity. There is no difference during the sensory age range (25-35 dph) or the period during which males exhibit sensorimotor refinement of learned songs (30-60 dph). By contrast, during the period of subadulthood prior to the completion of sexual maturity (60-80 dph) estradiol levels in NCM were elevated in males as compared to females. Numbers indicate sample sizes for each sex/age range. * $p < 0.05$

Figure 4.

The same data as presented in Fig. 3 were re-plotted here in regression format to examine age-dependent changes in males vs. females. A. In males, there was a significant, linear effect of age on NCM E2 levels, with age treated as a continuous variable in linear regression ($F_{(1,23)} = 30.718$; $p < 0.0001$; $R^2 = 0.57$). B. By contrast, in females there was no significant effect of age, treated as a continuous variable in linear regression ($F_{(1,23)} = 1.349$; $p = 0.268$; $R^2 = 0.10$). 'Mean Age' refers to the average age of the bird during the multi-day microdialysis sampling period.

A. Morning trial

B. Afternoon trial

Figure 5.

Levels of estradiol (E2) within NCM are responsive to song tutoring in the morning (A) but not afternoon (B). Both sexes exhibit a significant elevation in NCM E2 levels in the immediate period of silence following song tutor playbacks ('Post 1'), but this pattern is only evident in experiments conducted in the morning and not in the afternoon. Each time period is 60 min, all raw E2 levels have been normalized to 100% baseline ('Pre') to standardize sex- and age-dependent effects. * $p < 0.05$.

Figure 6.

Patterns of response to song exposure are entirely different between adult and juvenile males for E2 within NCM. Data from Fig. 5 for juvenile males are plotted again here to provide a direct visual comparison with data from adult males. For experiments conducted in the morning, while adult males exhibit a significant elevation in NCM E2 levels during song exposure, juvenile males exhibit a significant elevation in NCM E2 levels only following tutor song exposure. Each time period is 60 min. * $p < 0.05$.

Figure 7.

The variance in Wiener entropy of the songs produced by a subset of $n = 8$ microdialyzed juvenile males is elevated following tutor exposure in this study. Wiener entropy is an inverse measure of tonality of a song, and an increase in the variance in Wiener entropy has become established as an indicator of the incorporation and refinement of new song elements as young males practice new song syllables (see Tchernicovksi 2000, Shank and Margoliash 2009). Each x-axis bin is one day of singing, and the points plotted are the average entropy variance values for songs from that day. The dashed line indicates no change in entropy variance. * $p < 0.05$.

Figure 8.

No significant changes in NCM E2 levels were observed in a subset of microdialyzed juvenile zebra finches in this study that were exposed to the auditory playback of tutor song in the morning (alone, without visual stimuli). In combination with Fig. 5, these findings suggest that the combined sensory modalities of visual and auditory song tutoring together were associated with fluctuations in NCM E2 levels.

Table 1

Baseline estradiol levels for *in vivo* microdialysis in juvenile males in the lateral nidopallium ('misses') are significantly lower than levels within NCM for age-matched peers. Each value is mean pg/ml of estradiol $\pm$ S.E.M.

Probe placement	Baseline E2	Age range	N	P-value for difference
Lateral nidopallium	3.23 $\pm$ 0.78	37-80 days	5	< 0.0001
NCM (age-match peers)	24.17 $\pm$ 3.38	39-80 days	18	

Table 2

Estradiol levels do not change significantly in response to tutor playback for *in vivo* microdialysis ‘misses’ in the lateral nidopallium. Each value is mean pg/ml of estradiol $\pm$ S.E.M for N = 5 males. For statistical tests see text. All samples were collected in series to determine changes over time (i.e., ‘Pre 1’ is a sample collection 120-60 min prior to the tutor playback trial and ‘Pre 2’ is 60-0 min prior to the tutor playback trial).

Tutor experiment– lateral nidopallium					
	Pre 1	Pre 2	Tutor playback	Post 1	Post 2
Morning	2.41 $\pm$ 0.82	2.98 $\pm$ 0.46	4.60 $\pm$ 1.56	4.69 $\pm$ 1.33	3.27 $\pm$ 1.98
Afternoon	4.06 $\pm$ 1.31	5.27 $\pm$ 2.30	4.63 $\pm$ 1.48	3.44 $\pm$ 1.54	2.53 $\pm$ 1.09