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– DRAFT –

**Using Digital Images of the Zebra Finch Song System as
a Tool to Teach Organizational Effects of Steroid
Hormones: A Free Downloadable Module**

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ABSTRACT

Zebra finch song behavior is sexually dimorphic: males sing and females do not. The neural system underlying this behavior is sexually dimorphic, and this sex difference is easy to quantify. During development, the zebra finch song system can be altered by steroid hormones, specifically estradiol, which actually masculinizes it. Because of the ease of quantification and experimental manipulation, the zebra finch song system has great potential for use in undergraduate labs.

Unfortunately, the underlying costs prohibit use of this system in undergraduate labs. Further, the time required to perform a developmental study renders such undertakings unrealistic within a single academic term.

We have overcome these barriers by creating digital tools, including an image library of song nuclei from zebra finch brains. Students using this library replicate and extend a published experiment examining the dose of estradiol required to masculinize the female zebra finch brain. We have used this library for several terms, and students not only obtain significant experimental results but also make gains in understanding content, experimental controls, and inferential statistics (ANOVA and post-hoc tests). We have provided free access to these digital tools at <http://mdcune.psych.ucla.edu/modules/birdsong>.

Key words

cyberlearning, zebra finch, bird song system, digital learning, distance learning, hormones, neuroscience

INTRODUCTION

The bird song system has had a long record of yielding cutting-edge findings in neurobiology. Adult neurogenesis (Alvarez-Buylla, Theelen, & Nottebohm, 1988) and dramatic sex differences in the brain (Nottebohm & Arnold, 1976) were first described in this system. This system is still the focus of ongoing investigations on neurosteroids (Schlinger, Soma, & London, 2001), learning and memory (Nordeen & Nordeen, 2004; Troyer & Doupe, 2000), and genes and proteins involved in vocal learning (White, Fisher, Geschwind, Scharff, & Holy, 2006; Xie, London, Southey, Annangudi, Amare, Rodriguez-Zas, Clayton, & Sweedler, 2010). The song system is also a valuable model of both the genetic and hormonal bases of sexual differentiation of nervous systems (Agate, Grisham, Wade, Mann, Wingfield, Schanen, Palotie, & Arnold, 2003; Duncan, Jimenez, & Carruth, 2009; Grisham & Arnold, 1995).

The song system consists of a set of interconnected nuclei whose only known function is the learning and production of song (Figure 1). This system can be divided into song acquisition and song production pathways. The song acquisition pathway consists of nuclei in the anterior forebrain pathway—Lateral magnocellular nucleus of anterior nidopallium (LMAN), Nucleus dorsolateralis anterior thalami, pars medialis (DLM) and Area X, which is its proper name (nomenclature from Nixdorf-Bergweiler, & Bischof, 2007; and Reiner et al., 2004). Lesions in this pathway disrupt the acquisition (Scharff & Nottebohm, 1991; Bottjer, Miesner, & Arnold, 1984; Johnson & Bottjer, 1991; Nottebohm, 2005) and maintenance of song (Roy & Mooney, 2007; Williams & Mehta 1999). The production pathway consists of HVC (here used as a proper name), the Robust nucleus of arcopallium (RA), and hypoglossal nucleus (nXII) (nomenclature from Reiner et al., 2004). Lesions of this pathway result in a loss of song (Nottebohm, Stokes, & Leonard, 1976). Electrophysiological evidence suggests that song syllable timing is organized in HVC and the syllables' actual acoustic properties are organized in RA (McCasland, 1987; Suthers & Margoliash, 2002; Vu, Mazurek, & Kuo, 1994).

In most (but not all—cf. Brenowitz & Arnold, 1986) songbird species, the song behavior is sexually dimorphic with males singing more than females. Accordingly, in most species, the telencephalic song nuclei (Area X, HVC and RA) are dramatically larger in males than in females (Nottebohm & Arnold, 1976; Gurney & Konishi, 1980; Grisham & Arnold, 1995—see Figure 2). This sex difference can be altered in development via the organizational effect of steroid hormones, which determine aspects of the sexual phenotype irrespective of the genetic code carried on the sex chromosomes (cf. Breedlove & Hampson, 2002; Phoenix, Goy, Gerall, and Young, 1959). Exogenous steroid hormones, particularly estradiol, can masculinize the brain phenotype in developing female zebra finches (Gurney & Konishi, 1980; Grisham & Arnold, 1995; Grisham, Lee, Park, Mankowski, & Arnold, 2008) and is also known to masculinize mammalian brains in development (cf. Breedlove and Hampson, 2002).

The rich literature on the song system, the robust difference between the sexes in the size of song nuclei, and the dramatic masculinizing effect of hormones on this system's development (Gurney & Konishi, 1980; Grisham & Arnold, 1995) make the zebra finch song system valuable for demonstrating the effects of hormones on development and sex differences in brain morphology. In particular, undergraduate neuroscience, biological psychology, and animal behavior laboratories could profit by using this system. Nevertheless, studying this system requires significant investments: supporting a bird colony, purchasing good quality microscopes, microtomes, histological supplies for processing brains, and digital microscope cameras. Such extensive facilities and equipment requirements make studying the bird song system out of reach for many institutions. We have overcome these barriers by creating a library of digital images of the bird song system and distributing it at no cost to users at <http://mdcune.psych.ucla.edu/>.

This library consists of images of female zebra finch brains used in a previously published study (Grisham, Lee, Park, Mankowski, & Arnold, 2008), along with added images from control males. The females were administered different doses of estradiol at hatching, and their brains were examined and photographed in adulthood. Using this library, students not only repeat an

experiment examining the relationship between the dose of estradiol and the degree of masculinization in the song system, but also extend the original experiment because they quantify images from control males that were not part of the original experiment (Grisham *et al.*, 2008). Because of the robust sex differences and large treatment effects, even inexperienced students will obtain data yielding significant differences.

METHOD

Materials

All that is required for this laboratory is a computer. The image library—available for downloading at the above address—consists of the relevant images of Area X, HVC, and RA in control male and control female zebra finches, as well as females that received 5, 15, or 50 μg of estradiol (E_2) at hatching. These images were made from tissue prepared for Grisham, *et al.*, 2008, so students can refer to this article for procedural details (Pubmed Central ID# PMC2605609). The males came from other studies in which they were treated at hatching with either blank implants (Grisham & Arnold, 1995) or implants of indomethacin, a prostaglandin inhibitor like aspirin, that had no discernable effect on the song system (Borowski *et al.*, 2010). The male brains were prepared in an identical way to Grisham, *et al.*, 2008. Students quantify the size of song regions using NIH ImageJ, which is a free software package that can run on any platform (see NIH Image homepage in references).

Procedure

Students are given the following five specific objectives to focus their thinking and to help structure their journal-style laboratory report: 1) Although this module isn't an independent replication, it is still useful to see if students' measurement and re-analysis yields the sex differences in the song system established in the literature (Nottebohm & Arnold, 1976; Grisham & Arnold, 1995). We use this as an opportunity to underscore why this step is necessary to validate their techniques and findings. We point out that there is no need to try to explore the underlying basis of a phenomenon if one cannot repeat it with the same materials. 2) To probe the sensitivity of the female zebra finch song system to estradiol-induced masculinization. This study was originally motivated by another study that substantially reduced E_2 synthesis and found no impact on the masculine development of the song system (Wade *et al.*, 1996). Nonetheless, if masculinization processes were exquisitely sensitive to E_2 , the residual E_2 could have been sufficient to masculinize the system. 3) To discern whether masculinization is an all-or-none event once a threshold was reached, or whether it is proportional to the dose of estradiol. 4) To ascertain whether different parts of the song system show differential sensitivity to estradiol, which could provide some clues about song system development. 5) To determine whether our largest dose of estradiol will fully masculinize the song system in females to the same extent as males. We provide students with background readings (Breedlove & Hampson, 2002; Grisham & Arnold, 1995; Grisham *et al.*, 2008; Nottebohm, 2005) as well as background lectures (see <http://mdcune.psych.ucla.edu/modules/birdsong>) and ask them to formulate predictions based on the readings.

We use this module to teach about experimental procedures to avoid possible confounds. First, each student is assigned a total of five birds, one from each different treatment condition (control male, control female, 5 μg , 15 μg , and 50 μg E_2 females—see Figure 3). This balancing procedure prevents students' differential quantification style from potentially confounding the results (discussed further in the [Complete Users' Manual: Thorough Guide to Obtaining and Analyzing Data for Students and Instructors](#), which can be found on the website). We use this design consideration to illustrate that when extraneous variables are present that cannot be eliminated, an experiment can be designed so that they do not operate differentially across treatments and potentially confound the results. Secondly, students are kept blind to the sex and

treatment of the birds while they are making measurements of the song system, and we explain to students that this controls for bias.

Using NIH ImageJ, students make cross-sectional measurements to the nearest .01 mm of all the images of Area X, HVC, and RA. PDFs on the website, including the [Complete Users' Manual: Thorough Guide to Obtaining and Analyzing Data for Students and Instructors](#), and FAQs provide details on quantification, including pitfalls. Students can convert these measurements to volumes because they know the sampling interval between sections (see the Complete Users' Manual on our website).

Students combine their data, and ultimately several birds' data are represented in each treatment condition. A master spreadsheet containing all the data along with information about the sex and treatment of each bird is distributed to each student. (Instructors may access a spreadsheet by going to <http://mdcune.psych.ucla.edu/faculty> and setting up a free faculty account). Students are guided in analyzing the data using OpenStat (Miller, 2010) or VassarStats (Lowry, 2010), which are free statistical analysis packages available on the web (URLs in the references). Specifically, students need to decide what type of experimental design is employed and what analysis would be appropriate (a one-way ANOVA with 5 levels). We use this data analysis to introduce Analysis of Variance and post-hoc statistical tests and why they are appropriate to analyze our data. As a post-hoc test, we typically use Fisher's Least Significant Difference test, which OpenStat does automatically at $p < .05$ if a significant overall F ratio is obtained. (Details on our lesson on ANOVA and factors that can influence statistical power are available as part of the PowerPoint presentations (Lecture 3) available on our website <http://mdcune.psych.ucla.edu/modules/birdsong>.)

Students characteristically obtain remarkably good data and invariably find significant differences. Data from one section of our students is displayed in Figure 4. Students are assigned to write up a report in the form of a journal article and are guided through this process via discussion of the five objectives outlined above (also see PowerPoint slides in Lectures 3 and 4 on our website <http://mdcune.psych.ucla.edu/modules/birdsong>).

Initially, we taught this module by interpreting the data for students. Both formal and informal feedback suggested that this "spoon feeding" wasn't desirable. Rather, students indicated that they would appreciate the intellectual challenge of interpreting the data themselves, especially since they already had the five-question framework. Teaching the module without interpreting the data for students predictably led to significantly lower grades [$t(141) = 2.088$, $p < .05$], with higher variance in scores, but the absolute difference in mean scores was only 3.13%. The intellectual exercise seemed well justified.

Implementing the Bird Song System Module

This module works well with a class of twenty-four students but it is adaptable for smaller or larger enrollments. We devote three weeks of three-hour labs and three one-hour lectures to this module. Students are easily able to complete their measurements and data analyses in this time. When we assign five birds (one from each condition) to each student, it takes about three to five hours, maximum, for students to finish quantifying Area X, HVC and RA in all of their birds. (This time includes orienting students to the Image J quantification tool and collecting all of their data in a master spreadsheet.) Data analyses are accomplished fairly quickly, but it takes much longer to explain the rationale and meaning of the analyses. Analyzing data, explaining their purpose, interpreting the results, and demonstrating how to present data in a meaningful manner takes a couple hours, which we usually distribute between lab and lecture. (See PowerPoint slides from Lectures 3 and 4.) Finally, as we are going through this project, we train students to write a journal-style lab report, and this effort usually takes one to two hours, which can either be in lecture or lab (again, see PowerPoint slides from Lectures 3 and 4).

Our students are either Psychobiology or Neuroscience Juniors and Seniors. Most students have had a course in statistics, and some have had exposure to neuroanatomy. Some have had some exposure to discussions on hormones and behavior, but most have not. Most have had a course in which they learn how to draft a journal article report. In teaching this module, we have found it useful to review relevant neuroendocrinology, neuroanatomy, statistics, and discussions on formulating a write-up. Notably, we discovered that even when students have a background in statistics and writing journal-style reports, they still need a refresher tutorial. To assist faculty with these tutorials, we have provided PowerPoint presentations focusing on neuroendocrine background (Lectures 1 & 2), statistics (Lecture 3), and writing (Lectures 3 & 4). Thus, pre-requisite courses are probably not necessary if the instructor provides relevant background on these topics. We use the exercise to reinforce statistical and writing skills that the students acquire in other courses, but this exercise also could be used to introduce these skills.

From the instructor's point of view, this teaching module is easy to implement. In teaching this unit, we identified and remedied many obstacles to make it a better learning experience for both students and instructors. Many common problems are addressed in the Complete Users' Manual and the FAQ list on our website.

Possible shorter version of this module

Instructors could construct a module that would take less time but would still be informative by using fewer groups. For example, instructors could use the control females and control males along with the females treated with 50 µg in order to demonstrate the sex difference and show that masculinization can be achieved by administering E₂. Alternatively, instructors could use just the control males and control females to demonstrate the sex differences.

Assessing the effectiveness of this module

All assessment measures had IRB approval (UCLA IRB Exemption # 07-211). Efficacy of this module was assessed in two consecutive terms in lab classes composed of UCLA Neuroscience and Psychobiology majors. Both groups have very similar demographics and career ambitions. We administered a pre/post quiz including a scale that assessed understanding of the content, statistics, and experimental design with a subscale of items that assessed "inappropriate thinking biases" based on those presented by Stanovich (2009). Students also completed a Materials Evaluation questionnaire that included Likert scale items and an open-ended item to assess their experience with the module and their opinions about the materials provided. The open-ended question asked students to describe the purpose of the birdsong module from a learning standpoint. The pre/post test and Student Materials Evaluation can be found in the Supplementary Materials.

Because repeated testing itself can sometimes raise scores (Campbell and Stanley, 1963; Trochim *et al.*, 1986, 2006), we administered the posttest alone in a second sample of students in a subsequent term. All students received the Materials Evaluation questionnaire, and their responses on this questionnaire were combined across both samples.

RESULTS

We compared the posttest scores to the pretest scores separately on our two scales: the module-specific and "inappropriate thinking biases" scales. One item on the module-specific scale was discarded because of poor psychometric properties (item #15), which seemed to be poorly worded; the pattern of results was not appreciably altered by discarding this item. The posttest means on the module-specific scale, addressing content, statistics, and experimental design, were significantly higher than the pretest of the first sample of students, $t(111) = 17.78$, $p < 0.001$, and $t(148) = 10.74$, $p < 0.001$, respectively (Figure 5). The posttest means on the module-specific scale were not significantly different from each other, $t(148) = .118$, $p = .906$, suggesting

that the gains between the posttest and pretest reflected genuine increases in learning and reasoning skills and not just an artifact of repeated testing (Figure 5). Also, grades on the module were not significantly correlated with pretest scores, $r(110) = .06$, $p > .50$, suggesting that better performance was not correlated with better preparation before the module. Post-test scores did correlate with grades on the module in both terms, $r(36) = .37$, $p < .05$ and $r(110) = .33$, $p < .05$, providing convergent validity for our module-specific pre/post measure. Not surprisingly, scores on the four-item “inappropriate thinking biases” subscale (Items 19-22 on the pre/posttest—see supplementary materials) were uninfluenced by the module; students performed roughly at chance both before and after the module, and comparisons on this subscale were non-significant, all $ps > .50$ (data not shown).

Responses to the Likert scale items reflected a general enthusiasm for the module and showed that students believed that they were gaining in knowledge and skills. Students generally agreed that they had learned a lot about sexual differentiation of the brain and data analysis (Figure 6). Students also liked getting significant results and repeating and extending a published experiment. Most students also reported that using the digitized images was easy and they felt that they learned as much from the digitized images as they would have from tissue on slides. (Notably, they had a side-project that dealt with using actual tissue on slides, so they had an apt comparison).

Students responded to an open-ended prompt, “Please describe the purpose of using the Bird Song System module from a learning standpoint.” Responses were coded as content-related (“sexual differentiation of the zebra finch,” “role that estradiol plays on the female zebra finch system”), comprehension (“to learn,” “to see,” “give students a better grasp”), experimental method (“how a scientific study is produced from formulation of hypothesis to the data collection,” “learn how to conduct a thorough research experiment”), data analysis (“students learned about ANOVA tests, how to justify and draw conclusions from data,” “applying the statistics that I’ve learned...to “real” data, and build critical thinking skills by analyzing the data”), writing skills (“wrapping up all the ideas in a formal research article format,” “formatting of a proper paper”), hands-on experience, extended relevance (“applicable to that of the human brain,” “generalize to human beings,” “connected to actual behaviors,” “further studies in mammals.”), positive affect (“excellent,” “module did a great job,” “Overall, I liked this module!” “It was very fun and enjoyable...”), and negative affect, which included all negative comments, whether regarding clarity or effectiveness of the materials. Table 1 presents the frequency of each response category.

DISCUSSION

This lab experience illustrates the organizational effect of gonadal steroids on brain development (Breedlove and Hampson, 2002), which occurs in early life during a critical/sensitive period and persists for the lifetime of the individual (Breedlove and Hampson, 2002; Phoenix, Goy, Gerall, and Young, 1959). Estradiol was administered when the birds hatched, yet the effects of this early exposure were still manifest in adulthood when they were sacrificed. Although the masculinization of the genetic females’ song system is only partial, it is still impressive (see Figure 3).

In the course of this module, students learn how to do a careful image analysis, how to work with a large dataset, how to perform inferential statistics (an ANOVA with post-hoc tests) and how to interpret experimental findings. Further, they are exposed to control considerations to avoid possible confounds. The module-specific pre/posttest showed that students made significant gains in these domains (Figure 5). The significant correlations of module-specific posttest scores with grades suggest that the pre/post test was tapping the same domain as did our assessment of students’ write-ups. Our subscale of “inappropriate thinking biases” did not show any change as a function of instruction, and scores were roughly at chance in both the pretest and posttest. This latter result is not surprising—these items largely test common thinking and heuristic biases

(Stanovich and West, 2008), and these reasoning fallacies seem difficult to overcome. It was ambitious to hope that the short amount of experience with our module could reform such biases. Perhaps a different measure of critical thinking would have been more appropriate.

The open-ended item, which tapped students' impressions of the purpose of the module (Table 1), indicated that the experience enhanced students' understanding of experimental methodology and design, data analysis, and writing skills as well as content. Although affect wasn't explicitly queried in the open-ended item, positive comments outweighed negative by a ratio of over 5:1 (Table 1).

The Likert-scale questionnaire indicated that students agreed that they had learned a lot about content and data analysis (Figure 6). Further, students appreciated being able to obtain significant differences in their data. One advantage to this lab is that effect sizes and sex differences are so large that even inexperienced students can obtain data that will yield significant differences. In teaching this module across several terms, students have never failed to obtain data that yielded significant differences: students' data usually comes out as it did in Figure 4. Thus, students obtain the sex differences described in the literature and can relate their results to published studies. Students find that although the female song system is not exquisitely sensitive to estradiol—5 μg never masculinizes—usually 15 μg is sufficient to masculinize the song system. Generally, students find that masculinization maximizes with a dose of 15 μg and doesn't increase with a dose of 50 μg . Further, 50 μg , which is a massive dose, does not completely sex-reverse the song system. Usually, different parts of the song system don't show differential sensitivity to estradiol, but because this is an inquiry-based lab, students' data don't always come out the same way (see the Complete Users' Manual on the website for a discussion of anomalous results).

Students reported that using the digitized images was easy, and they felt that they learned as much from the digitized images as they would have from tissue on slides. Notably, we had students perform a side-project in which they sectioned a zebra finch brain and then tried to determine the sex of their "mystery" bird by examining the song system. Although students seem to enjoy slicing and staining the actual tissue, we have found that using the image library is far superior to using the actual tissue on slides. When using the actual tissue, students and even graduate teaching assistants were observed wandering around the lower brainstem trying to find the song nuclei that are in the forebrain. Clearly, the image library focuses neophytes on the task better than using the actual tissue.

Our data suggest that this learning experience was an enjoyable and valuable one from the students' perspective. We have created the materials so that instructors can easily adopt the module. Further, no specialized equipment is required to provide this experience to students, only computers connected to the Internet. Due to their digital format, these materials can be incorporated into a standard classroom, a laboratory setting, or even distance learning.

All materials required for teaching this module, including the image library, the Complete Users' Manual, PDF handouts, PowerPoint slides with and without voice-over commentary, and links to relevant websites, as well as lectures on the material, are available for free at <http://mdcune.psych.ucla.edu/modules/birdsong>. Grading keys/rubrics, keys to the treatment group for each bird, and instructor-specific materials also can be obtained for free by accessing <http://mdcune.psych.ucla.edu/faculty/create-a-faculty-account> and setting up a faculty account.

FIGURES AND CAPTIONS

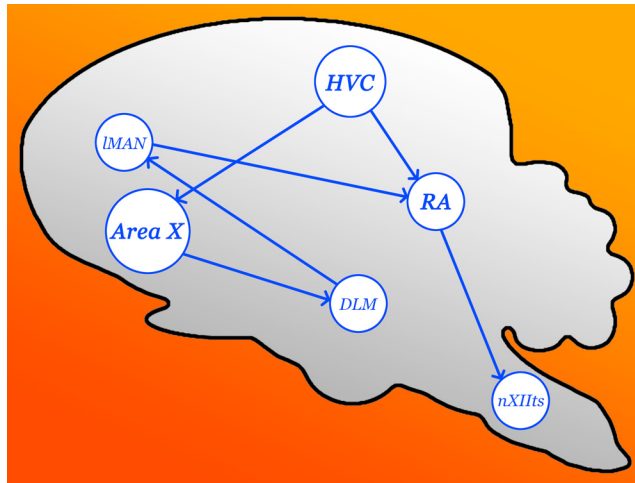


Figure 1. Semi-schematic of a sagittal view of a birdsong brain highlighting the relationships among the song nuclei. RA, IMAN, Area X, and HVC are all telencephalic nuclei. RA stands for Robust nucleus of the Archopallidum, IMAN stand for lateral Magnocellular Nucleus of the Anterior Nidopallium, and HVC and Area X are used as proper names (see Reiner et al., 2004). RA, IMAN, and HVC can be considered as roughly homologous to aspects of the mammalian cortex. Area X is a part of the basal ganglia and is also part of the forebrain. DLM and nXII are in the brainstem and stand for Dorsolateral Medial nucleus of the thalamus and the nucleus of nerve XII.

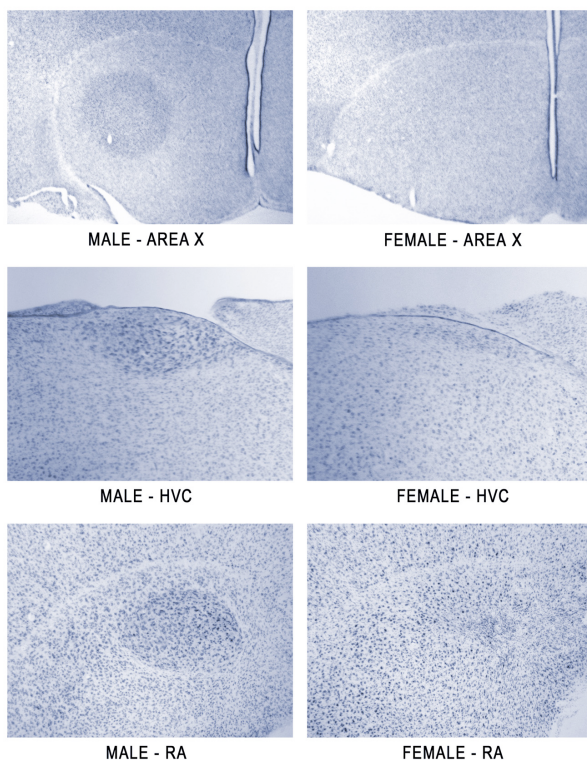


Figure 2. Side-by side comparisons of male and female Area X (females don't have an Area X), HVC, and RA. These images are from our library. Each individual would have multiple images from all sections across the rostral-caudal extent of a given song nucleus, so that students can determine the volume.

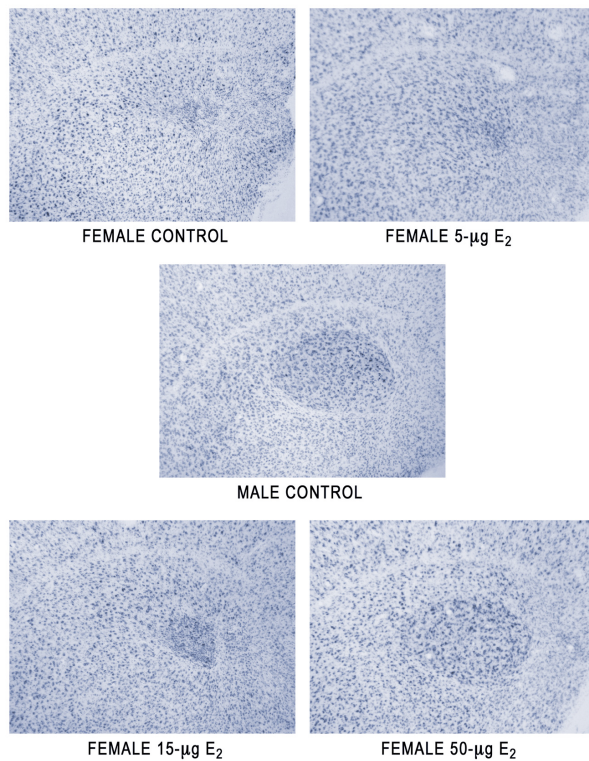


Figure 3. RA in a single section from each of our groups. Differences are quite evident and easy to quantify. (Again, students quantify multiple sections for each nucleus of each bird.)

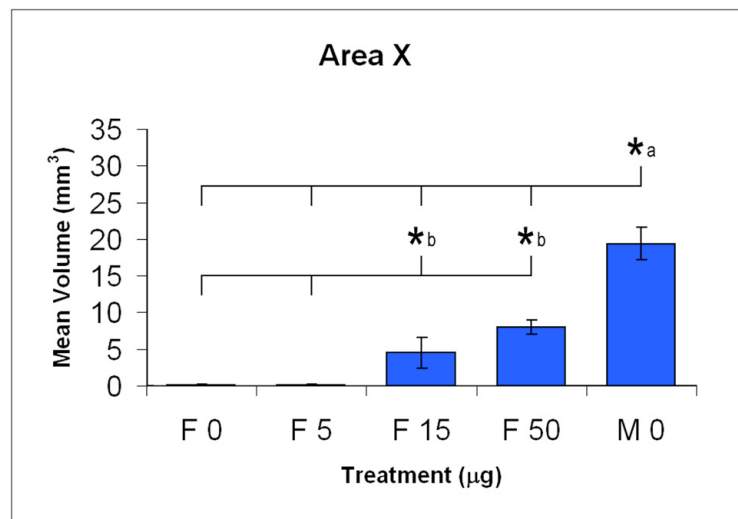


Figure 4. Mean Area X volume ($\pm$ SEM) as a function of dose of E_2 (0, 5, 15, & 50 μ g) and sex (M or F). Letter "a" signifies different from all other groups, letter "b" and brackets signify difference of 15 and 50 μ g doses from 5 and 0 μ g, Fisher's Least Significant Difference test. Data were obtained by our students; only Area X data is shown here, but HVC and RA data show a similar pattern of significant differences.

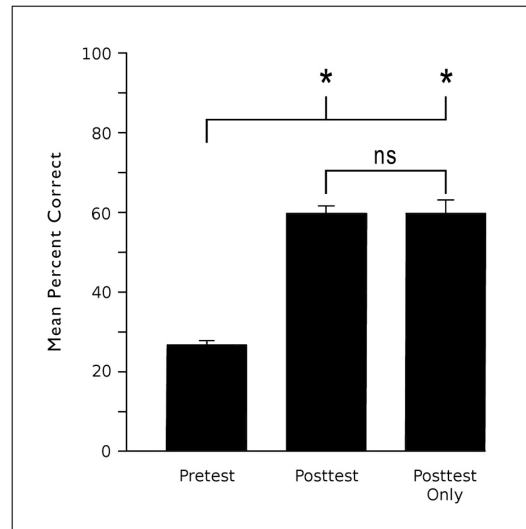


Figure 5. Mean percent correct ($\pm$ SEM) on pretest compared to posttest scores from two different academic terms—in posttest-only condition, pretest was not administered. Asterisk indicates $p < 0.001$, ns = nonsignificant.

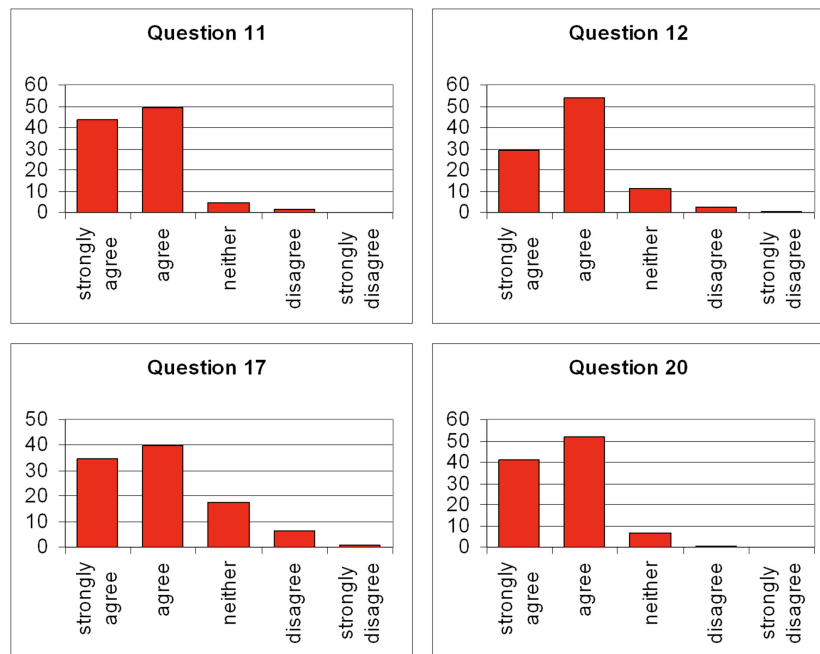


Figure 6. Responses to selected questions on our Likert scale—percent of total students in both samples combined ($n=153$) as a function of scale point (full set of responses and questions in the supplementary materials). Question 11—I learned a lot about sexual differentiation of the brain through the Bird Song System module. Question 12—I learned a lot about analyzing data through the Bird Song System module. Question 17—I felt like I learned as much using the digitized images as I would have using tissue on slides. Question 20—I appreciated being able to jump right in and collect data.

Category	Frequency
Comprehension	100
Content	89
Experimental method	57
Data analysis	40
Writing skills	36
Hands on experience	24
Extended relevance	12
Positive affect	25
Negative affect	4

Table 1. Categorization of responses to the open-ended question “Please describe the purpose of using the Bird Song System module from a learning standpoint.” A given student’s responses can be coded in more than one category. (N = 136 students providing data on this item).

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