NEUROENDOCRINE CONTROL OF SONG IN THE

DARK-EYED JUNCO (*JUNCO HYEMALIS*)

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A

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ABSTRACT

This dissertation includes three discrete projects addressing various aspects of the neuroendocrine control of song in the Dark-eyed Junco (*Junco hyemalis*), a migratory songbird. Specifically, the roles of testosterone, photoperiodic condition, opioids, and age were investigated with respect to song production and neural plasticity in the regions of the brain that control song (vocal control regions, VCRs). I found that, in males, photoperiodic condition and testosterone interact to regulate seasonal VCR volume plasticity, whereas testosterone alone controls song production. The opioid system is probably not involved in VCR plasticity or song production, but is indicated to play a role in song learning or auditory processing. Finally, VCR volumes and song production do not differ with age in photostimulated adult male juncos.

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I. INTRODUCTION

Why Study Birdsong?

The modern study of birdsong began with the work of William Thorpe (1958, 1961). He showed that chaffinches (*Fringilla coelebs*) collected as nestlings and reared in the laboratory in isolation from conspecific adult males produced very abnormal songs. However, if these birds were exposed to taped recordings of wild chaffinch songs, they eventually produced normal songs that closely matched those of the recordings. These studies showed for the first time that young birds must learn the song of their species by listening to adult conspecifics. Peter Marler, one of Thorpe's students, greatly expanded on this early work. Marler and his colleagues demonstrated that song learning is characterized by early sensitive periods, that birds have an innate predisposition to learn the song of their species, and that local geographic song dialects exist (Marler, 1970, 1976). A student of Marler's, Masakazu Konishi, showed that birds must be able to hear themselves sing to develop song normally (Konishi, 1965). Finally, Fernando Nottebohm, also a student of Marler's, showed that the peripheral control of song production is lateralized. Nottebohm and his colleagues subsequently identified the neural circuits in the avian forebrain that control song behavior (Nottebohm *et al.*, 1976, 1986). This important discovery paved the way for many investigators who have henceforth contributed to our understanding of song behavior and its neural control.

In general, song serves two main functions (Catchpole and Slater, 1995; Kroodsma and Miller, 1996). In many species, song is used to declare a territory from

which other birds are aggressively excluded, as shown by the fact that muting birds decreases their ability to deter intrusions by other birds. Both males and females may use song in this context. Song may also be used by males to attract females, as well as to stimulate the female's reproductive behavior and physiology. In bird species residing in the tropics, song is often used for territorial defense throughout the year. In temperate-zone bird species, song used either in the territorial or mating context is produced at higher rates during the breeding season, and at lower rates or not at all outside the breeding season.

The birdsong system, therefore, offers several advantages as a model for identifying the neural mechanisms that underlie an observable, biologically relevant behavior (reviewed in Brenowitz *et al.*, 1997). First, song is a learned behavior that is controlled by discrete neural circuits. Because there are distinct phases in the development of song, with well-defined sensitive periods, one can relate the ontogeny of song behavior to the development of the underlying neural circuits. Song behavior and the associated neural circuits are also sexually dimorphic in most species, providing researchers with a valuable model for investigating the neural basis of a sexually dimorphic behavior. Gonadal steroids have pronounced effects on the development and adult function of the song control circuits, as well as on song behavior. There is extensive plasticity of the adult song system, including ongoing neurogenesis and seasonal changes in morphology. Finally, there is pronounced species diversity in different aspects of song behavior, including the timing of vocal learning, sex patterns of song production, number of songs that are learned, and seasonality of song behavior.

This diversity provides opportunities for comparative studies of the song system. Altogether, these attributes make the song system a valuable model for studying the neural acquisition and development of communication in higher vertebrates.

Song Development

Singing behavior develops in phases as a bird ages, and the timing of these phases differs among species (reviewed by Nottebohm, 1993; Marler, 1991). Birds memorize a song template shortly after hatching during the "sensory phase". Konishi (1965) showed that birds must learn their song from an adult tutor, since birds deafened prior to song memorization or raised in acoustic isolation do not develop normal adult songs. The sensory phase is followed with a "plastic song phase" during which the birds attempt to match their own vocalizations to those of the template (reviewed by Nottebohm, 1993). Auditory feedback is as essential here as during the sensory phase, because birds must hear their own song in order to compare it to the stored template (Konishi, 1965). Finally, this plastic song develops into full adult, "crystallized" song (Nottebohm, 1993). Maintenance of adult song is also somewhat dependent on auditory feedback. For example, birds deafened after the time of song crystallization will eventually show song degradation (Nottebohm *et al.*, 1976; Nordeen and Nordeen, 1992). Therefore, the memorization, vocalization, and auditory processing of song are tightly intertwined, and the vocal control system plays an important role in all of these aspects of song acquisition and production.

Song Control Circuits and VCRs

In oscines, song behavior is regulated by a discrete network of interconnected brain regions collectively called the vocal control system (Nottebohm *et al.*, 1976, reviewed in Konishi, 1994; Figure 1). The motor pathway controls the production of song, and some portion of this circuit presumably participates in learning. This circuit consists of projections from the thalamus nucleus Uva and the neostriatal nucleus NIf to the neostriatal nucleus HVc (higher vocal center). HVc projects to the robust nucleus of the archistriatum (RA) in the forebrain, and RA projects both to the dorsomedial part of the intercollicular nucleus in the midbrain and to the tracheosyringeal part of the hypoglossal motor nucleus in the brain stem (nXIIts). Motor neurons in nXIIts send axons to the muscles of the sound-producing organ, the syrinx. Neuronal activity in the premotor nuclei HVc and RA is synchronized with the production of sound by the syrinx (Vicario, 1991; Margoliash, 1997). If nuclei in the motor pathway are inactivated, a bird may adopt appropriate posture and beak movements, but does not produce song (Nottebohm *et al.* 1976).

The second, or anterior forebrain, pathway is essential for song learning and recognition (reviewed by Doupe and Solis, 1997; Margoliash, 1997). This pathway consists of projections from HVc to Area X, then to nucleus DLM in the thalamus, from DLM to the lateral portion of the magnocellular nucleus of the anterior neostriatum (lMAN), and finally to RA. In addition, lMAN neurons that project to RA send collaterals to Area X, thus providing the potential for feedback within this pathway. Inactivation of lMAN, DLM, or Area X in adults apparently does not disrupt previously

crystallized song, whereas the same lesions in juveniles prevent the development of normal song (Bottjer *et al.*, 1984; Sohrabji *et al.*, 1990; Scharff and Nottebohm, 1991; Halsema and Bottjer, 1992). Juvenile males with lesions of Area X persist in producing songs that are plastic in structure, as though they are unable to crystallize. Another lesion study found that HVc, which receives auditory input from the telencephalic Field L (Kelley and Nottebohm, 1979), is necessary for female canaries (*Serinus canaria*) to discriminate between conspecific and heterospecific songs (Brenowitz, 1991). Neurons in all telencephalic VCRs (Area X, lMAN, HVc, and RA) respond to auditory stimuli, and many are selective for the bird's own song, a characteristic that appears to develop during the plastic song phase (Margoliash, 1986; Margoliash and Fortune, 1992; Volman, 1993; Doupe, 1997).

Seasonal Plasticity and Testosterone

VCRs undergo pronounced seasonal changes in morphology in the adults of several songbird species. These seasonal changes may be related to seasonal changes in the quality or quantity of song production and may serve as a substrate for seasonal modifications of song in species that change their song from year to year (Nottebohm, 1981; Nottebohm *et al.*, 1986; Smith *et al.*, 1995a; Smith *et al.*, 1995b). Several attributes of certain song nuclei change seasonally, including the volume, size, density and number of neurons, and incorporation and survival of new neurons (Nottebohm, 1981; Kirn *et al.*, 1989; Brenowitz *et al.*, 1991, Smith *et al.*, 1995b; Johnson and Bottjer, 1995; Alvarez-Buylla *et al.*, 1990; Nottebohm *et al.*, 1994). One or more of these

seasonal changes have been found in several of the different song nuclei, including HVc, RA, and Area X.

Photoperiod is one of the most important environmental cues regulating seasonal changes in reproductive physiology and behavior (reviewed in Wingfield and Kenagy, 1991). Long days (LD) in spring initiate gonadal recrudescence and a resulting increase in plasma concentrations of gonadal steroids (Wingfield and Farner, 1980; Farner, 1986). VCR volumes are also larger at this time than after the breeding season, when birds are photorefractory and have low plasma T levels (Bernard and Ball, 1995; Kirn *et al.*, 1989; Nottebohm, 1981). Most of the song nuclei that undergo seasonal changes contain intracellular receptors for gonadal steroids (Arnold *et al.*, 1976; Gahr, 1990; Balthazart *et al.*, 1992; Brenowitz and Arnold, 1992; Smith *et al.*, 1996). In free-living birds, it is therefore likely that LD increases the size of the song nuclei by increasing circulating concentrations of T, which then acts directly or via estrogenic metabolites on steroid receptors in the song nuclei. In support of this, comparable changes also occur in captive songbirds exposed to breeding *versus* nonbreeding photoperiods or testosterone (T) concentrations (Nottebohm, 1981; Kirn *et al.*, 1989; Brenowitz *et al.*, 1991; Smith *et al.*, 1995b).

Photoperiod itself

Aside from inducing a vernal increase in plasma T levels, LD may either act on the VCRs *via* steroid-independent mechanisms or modulate the responsiveness of the song nuclei to seasonal changes in gonadal steroid levels. Photoperiod has been shown to

have steroid-independent effects on neural and behavioral plasticity in other systems (Steel and Hinde, 1972; Campbell *et al.*, 1978; Morin and Zucker, 1978; Meirnicki *et al.*, 1990; Lee *et al.*, 1995). However, there is also evidence that photoperiod modulates the actions of T on both song behavior and anatomical attributes of the VCRs (Smith *et al.*, 1997a,b; Nowicki and Ball, 1989; DeVoogd *et al.*, 1985; Clower *et al.*, 1989). Finally, photoperiodic condition may have effects on the VCRs as well. In many birds, continued exposure to LD during the summer does not maintain T levels and gonadal growth, but instead leads to a spontaneous collapse in gonad size and endocrine secretion, and a state of insensitivity to LD called photorefractoriness (Farner *et al.*, 1983; Wilson and Donham, 1988; Nicholls *et al.*, 1988). Normally, decreasing daylengths in the fall and early winter break this insensitivity to LD, thus making birds able to respond to LD again, or photosensitive (Nicholls *et al.*, 1988). Studies by Nowicki and Ball (1989) and DeVoogd *et al.* (1985) indicate that T-induced song production is modulated by photoperiodic condition, and it is possible that the VCRs are affected in the same way.

Opioids

Although gonadal steroids are important, they do not control all aspects of sexual differentiation, song development, and song production (reviewed by Arnold *et al.*, 1996). Many other neurochemicals and/or their receptors have been found in the VCRs and these substances may play some role in the vocal control system (Ball *et al.*, 1988; Casto and Ball, 1994; Soha *et al.*, 1996; Kimpo and Doupe, 1997). Studies on adult birds have found that VCRs contain both opioid peptides (Ryan *et al.*, 1981; Ball *et al.*, 1988,

1995; Bottjer and Alexander, 1995; Deviche and Gunturkun, 1992; Carrillo and Doupe, 1995) and their receptors (Gulledge and Deviche, 1995, 1999). In chicks, the opioid system is involved in the control of distress vocalizations (Panksepp *et al.*, 1978, 1980). In addition, opioids influence cell plasticity and neuronal survival (Meriney *et al.*, 1991; Zagon and McLaughlin, 1987; Hammer and Hauser, 1992) and interact with gonadal steroids (Bhanot and Wilkinson, 1984; Nikolarikis *et al.*, 1986; Forman and Estilow, 1988; Deviche, 1992). Altogether, this information indicates that the opioid system may play a role in some aspect of VCR plasticity and/or song behavior.

Age

It has been shown that year-classes differ in reproductive morphology and circulating levels of T in free living Dark-eyed Juncos (Deviche, Wingfield, and Sharp, in press). Year-class differences in CP width, plasma T, and testes weight also occur in free living Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*) (Morton *et al.*, 1990). In both cases, older adult males had higher levels of plasma T, larger CP widths, and heavier testes than their younger counterparts. As of yet, no study has investigated whether these differences also occur in song production or VCR volumes. Because song production and VCR volumes are influenced by T, it is possible that year-class differences in plasma T levels will translate to differences in song production and VCR volumes as well.

Thesis Objectives

There are three broad goals of this thesis, each represented by one chapter. The first is to determine the relative contributions of photoperiodic condition and testosterone to song production and VCR plasticity. The second goal is to investigate the role of opioids in the seasonal variation in song production and VCR volume plasticity in Dark-eyed Juncos. Finally, the third goal is to determine whether year-class differences in the reproductive physiology of adult male juncos are also apparent in VCR volumes and song production. In all of these studies, adult male Dark-eyed Juncos (*Junco hyemalis*) were our subjects. Juncos have been used for a long time to study photoperiodism, including the coordination of reproductive activity with changes in photoperiod (Rowan, 1925). Juncos are locally abundant, relatively easy to capture, and easily cared for in captivity. Unlike studies on canaries and zebra finches, any information gained about the vocal control system and song behavior in juncos can be applied toward understanding the species in the natural environment.

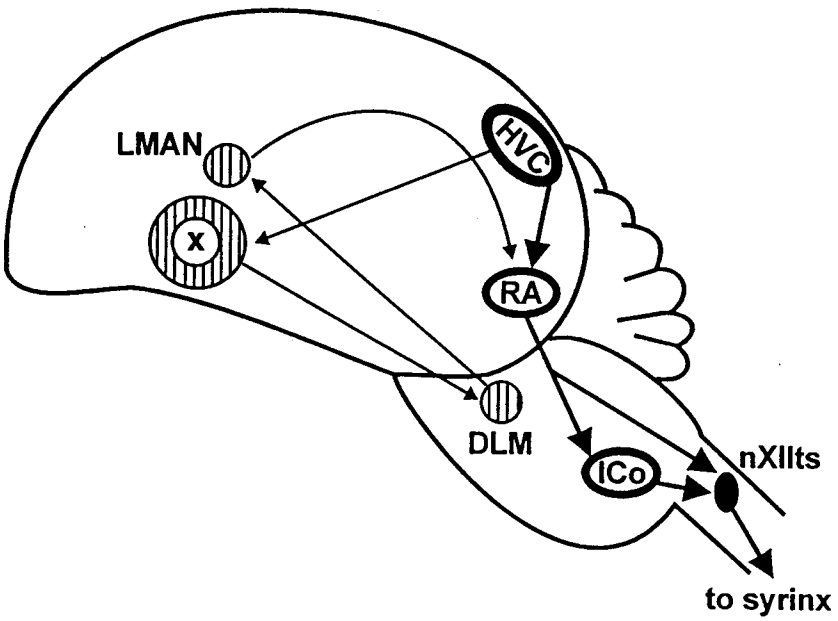

Figure 1. Diagram of the avian vocal control system. Hatched regions form the anterior forebrain pathway. Regions outlined in black form the motor pathway. (Adapted from Gulledge and Deviche, 1998)

II. EFFECTS OF TESTOSTERONE AND PHOTOPERIODIC CONDITION ON SONG PRODUCTION AND VOCAL CONTROL REGION VOLUMES IN ADULT MALE DARK-EYED JUNCOS (*Junco hyemalis*)

(As submitted to *Hormones and Behavior* by Dloniak and Deviche)

ABSTRACT

In seasonally breeding male songbirds, song learning and production are controlled by an interconnected set of brain regions (vocal control regions, VCRs) that exhibits seasonal neuronal plasticity throughout adulthood. Several of the VCRs contain androgen receptors and are androgen-sensitive throughout adulthood. In the present work, we determined whether exposure of photosensitive adult male Dark-eyed Juncos (*Junco hyemalis*) to long days influences VCR volumes and song production independent of plasma T levels by independently manipulating T and photoperiodic condition in castrated (Cx) adult males. We also compared the influence of T administration on song expression and VCR volumes in photosensitive, photostimulated, and photorefractory adult male juncos. Exposing Cx photosensitive males to LD enhanced their HVc volumes and these volumes were not further increased by concurrent T treatment. HVc and Area X were smaller in photorefractory than photostimulated males, but HVc increased in response to T treatment in photorefractory males. T treatment to SD-exposed photosensitive males increased HVc, but not Area X, MAN, or RA volumes. Only T-treated males sang and this treatment was equally effective behaviorally when given to Cx photosensitive and photostimulated or photorefractory juncos. Thus, photostimulation can increase HVc volumes maximally, but large volume maintenance in these birds

apparently requires elevated plasma T levels. Further, the stimulating influence of LD exposure on HVc volumes is insufficient to induce song in the absence of elevated plasma T levels.

INTRODUCTION

In most bird species breeding at middle and high latitudes, timing of reproduction is regulated by seasonal changes in photoperiod. Long days (LD; > approximately 12 hours of light per day) in the spring cause photosensitive birds to become photostimulated, thereby initiating gonadal recrudescence and a resulting increase in circulating gonadal steroid levels (Wingfield and Farner, 1980; Farner, 1986). At the end of the breeding season, when days are still longer than the threshold necessary to stimulate the reproductive system in spring, birds become photorefractory, at which time secretion of gonadal steroids decreases and the reproductive system is no longer responsive to LD (Nicholls, Goldsmith, and Dawson, 1988). Finally, the very short days of early winter (SD; < approx. 12 hours of light per day) terminate the photorefractory period, thereby restoring photosensitivity in preparation for the next breeding season (Nicholls *et al.*, 1988; Wilson, 1992). The physiological changes taking place during the reproductive period are associated with profound behavioral modifications. Most oscines sing at a high rate during the breeding season, when they are photostimulated and plasma testosterone (T) levels are high, and singing stops or decreases when plasma T levels drop after the breeding season, when birds are photorefractory (Marler, Peters, and Wingfield, 1987; Nottebohm, Nottebohm, Crane, and Wingfield, 1987). In several

species, singing is diminished or eliminated by castration, and subsequent T treatment reinstates the behavior (Arnold, 1975; Heid, Guttinger, and Prove, 1985; Harding, Walters, Collado, and Sheridan, 1988).

In oscines, both song learning and production are controlled by an interconnected set of brain regions (vocal control regions or VCRs) collectively called the vocal control system (Nottebohm *et al.*, 1976; reviewed by Konishi, 1994). This system includes the high vocal center (HVc), Area X of the parolfactory lobe, magnocellular nucleus of the anterior neostriatum (MAN), and robust nucleus of the archistriatum (RA). Area X and MAN are essential for song learning (Nottebohm *et al.*, 1976; Bottjer, Meismer, and Arnold, 1984; Sohrabji, Nordeen, and Nordeen, 1990; Scharff and Nottebohm, 1991), whereas HVc and RA are necessary for song expression (Nottebohm *et al.*, 1976).

The vocal control system exhibits neuronal plasticity throughout adulthood in many species (Nottebohm *et al.*, 1976; Nottebohm, Nottebohm, and Crane, 1986; Smith, 1996; Brenowitz, Baptista, Lent, and Wingfield, 1996; Gulledge and Deviche, 1997). In seasonally breeding adult songbirds, VCR volumes are larger during than after the breeding season (Smith, 1996; Brenowitz *et al.*, 1996, Gulledge and Deviche, 1997). Changes similar to those observed in free-living birds occur in captive birds exposed to breeding *versus* nonbreeding photoperiods or T concentrations (Nottebohm, 1981; Brenowitz, Nalls, Wingfield, and Kroodsma, 1991; Smith, Brenowitz, Wingfield, and Baptista, 1995; Gulledge and Deviche, 1997). The effects of T on VCR volumes and singing are presumably mediated by androgen receptors located in HVc, RA, and MAN (Arnold, Nottebohm, and Pfaff, 1976; Smith, Brenowitz, and Prins, 1996). Although

androgen receptors are not present in Area X, this region receives projections from HVc, suggesting that the effects of T on this region are mediated by HVc (Arnold, 1980; Gahr, 1990). MAN also projects to Area X and may play a role in the effects of T on this region as well (Nixdorf-Bergweiler, Lips, and Heinemann, 1995; Vates and Nottebohm, 1995).

In addition to stimulating T secretion, photoperiod itself has gonadal androgen-independent effects on VCRs. Tree Sparrows (*Spizella arborea*) that are castrated prior to photostimulation show increases in HVc, Area X, and RA volumes in response to LD exposure (Bernard, Wilson, and Ball, 1997). In Gambel's White-crowned Sparrows (*Zonotrichia leucophrys gambelii*), Smith, Brenowitz, Beecher, and Wingfield (1997a) found a small but significant steroid-independent stimulatory effect of photostimulation on the volume of HVc and the size of RA neurons. In adolescent photorefractory male Dark-eyed Juncos (*Junco hyemalis*), exposure to LD increases the volumes of Area X, HVc, and RA despite low plasma T concentrations (Gulledge and Deviche, 1998). Finally, Kirn and Schwabl (1997) have shown that seasonal changes in photoperiod regulate neuron death rate in adult male canaries independent of changes in gonadal steroid levels.

The actions of T on VCR volumes and song behavior are modulated by photoperiodic condition. Nowicki and Ball (1989) showed that the song rate of photosensitive T-treated male Song Sparrows (*Melospiza melodia*) increased following transfer from SD to LD, even though this transfer did not increase plasma T levels. In the same study, the authors concluded that T treatment is equally effective in inducing song

in both photorefractory and photosensitive birds exposed to LD. Bernard and Ball (1996) found that HVc volume was larger in T-treated photostimulated than in intact photosensitive or T-treated photorefractory adult male European Starlings (*Sturnus vulgaris*). T administration to castrated photostimulated juncos also maintains large HVc and Area X volumes (Gulledge and Deviche, 1997), suggesting that large VCR volume maintenance in photostimulated birds depends on gonadal steroids.

No previous study has compared the effects of T treatment on song production and VCR volumes between photosensitive, photostimulated, and photorefractory males concurrently. In the present work, we independently manipulated photoperiodic condition and T treatment in adult male Dark-eyed Juncos, a photoperiodic, high-latitude breeder used in previous song system research (Gulledge and Deviche, 1997, 1998). We investigated the effects of T treatment on song rates across photoperiodic conditions, the relative importance of T treatment and photoperiodic condition in VCR volume plasticity, whether there is a relationship between VCR volume and song rate, and whether the VCRs of castrates undergo seasonal changes in volume.

MATERIALS AND METHODS

Experimental design

Experiment 1: Photosensitive Males. We collected 48 adolescent male Dark-eyed Juncos from a wild population near Fairbanks, Alaska (65°N, 148°W), in September, 1997, using seed-baited Potter traps. Birds were brought into captivity and housed in groups of 8-12 in indoor group flight cages. They were exposed to SD (8L:16D; lights on

at 0800 hrs) until March 11, 1998 (Figure 1). At this time, birds were moved to individual cages that were visually, but not acoustically, isolated from one another. Forty birds were bilaterally castrated under complete anesthesia *via* methoxyflurane inhalation (Metofane; Pitman-Moore Inc., Mundelerin, IL) between March 18 and 20. At this time, males either remained exposed to SD (n=16) or were transferred to a photostimulating light regime (n=24). Photostimulated birds were gradually exposed to increasingly longer days, by adding one hour of light per day until 20 hours of light were reached (LD; 20L:4D; lights on at 0400 hrs; Figure 1). The remaining eight birds were laparotomized and did not receive implants (see below). They were also transferred to LD to serve as a photostimulated intact group (STIM-I). On March 26, eight SD and eight LD (SENS-T and STIM-T) birds received two subcutaneous T-filled Silastic implants. T implants consisted of a 10 mm length of Silastic tubing (Konigsberg Instruments, Inc., Pasadena, CA; internal diameter, 1.5 mm; external diameter, 2 mm) filled with crystalline T (Sigma Chemical Co., St. Louis, MO) and sealed with silicone adhesive (Dow Corning, Midland, MI). All implants were incubated in a physiological saline solution at 37° C for 24 hours prior to implantation to initiate release of the steroid. Another eight SD and eight LD (SENS-C and STIM-C) birds received empty, control implants. Birds remained exposed to their respective photoperiods for the remainder of the experiment. All SD birds and 16 LD birds (STIM-C and STIM-T) were killed on May 6 or 7. The remaining eight LD castrated birds were kept until they had become photorefractory (REF-CX), as determined by the onset of prebasic molt (Morton, King, and Farner, 1969; Dawson, 1997; Dawson and Sharp, 1998), and were killed on July 14 (approximately two weeks

after the onset of molt). At the time of sacrifice, body cavities were inspected to ensure that castrations were complete. Throughout the study birds received Mazuri parrot and small bird pelleted food (PMI Nutrition Int., St. Louis, MO) and Avi-Con vitamin treated-water (Vet-A-Mix Inc., Shenandoah, IA) *ad libitum.*

Experiment 2: Photorefractory Males. During the second half of June, 1998, when birds are naturally exposed to constant light, we used mist nets and conspecific song playbacks to collect 22 adult male juncos. Birds were housed in visually isolated individual cages, received food and water *ad libitum* as in the first experiment, and they continued to be exposed to LD (20L:4D; lights on at 0500 hrs). They were checked periodically for the onset of molt as an indicator of photorefractoriness. All birds were molting by July 14. On July 21, 12 birds received T implants (REF-T) as described in the first study. Duration of T treatment was identical to that of experiment 1. The remaining 10 males received empty implants (REF-C). Birds were kept on LD until they were killed on September 2 or 3.

Blood Samples and Testosterone Assay

During each study, blood samples were collected from the left alar wing vein 12 or 13 and 32 or 33 days after hormonal treatments began. Samples were immediately centrifuged and plasma was drawn off and stored at -20° C until assay. Aliquots of plasma (25 µl) were assayed for total T by radioimmunoassay using a commercial coated tube ^{125}I kit (Diagnostic Products Corp., Los Angeles, CA). This assay has been used

previously for measuring T in Dark-eyed Juncos (Gulledge and Deviche, 1998) and is both sensitive (lower detection limit: 10 pg/tube) and specific (cross-reactivity: 3% with dihydrotestosterone, 0.02% with estradiol). All samples were assayed in duplicate in two series. The intra- and inter-assay coefficients of variation were 5.8% and 11.6%, respectively.

Morphological Measures

In order to assess the effectiveness of T implants, we measured cloacal protuberance widths (CP; a T-sensitive secondary sex characteristic: Schwabl and Farner, 1989; Deviche, 1992) to the nearest 0.1 mm with calipers 12 and 33 days after implantation. Gonads of photorefractory birds were collected and weighed to the nearest mg at the time of sacrifice.

Song Rate

In each study, the average song rate of each bird was quantified twice: between 7 and 10 and between 28 and 31 days after the onset of T administration. At both times, the same observer recorded the number of times each bird sang during two 30 min periods. Time periods were randomly assigned to each bird, and all observations were made between 0600 and 1130 hrs. The two counts of number of songs produced by each individual were then averaged at each time.

Brain processing and VCR volume measurement

All birds were killed by *in vivo* perfusion. Briefly, males were anesthetized by a xylazine/ketamine pectoral injection (0.032 mg xylazine (Loyd Laboratories, Shenandoah, IA) and 1.6 mg ketamine (Phoenix Pharmaceutical Inc., St. Joseph, MO) per 0.2 ml sterile saline), followed by methoxyflurane inhalation. Once completely anesthetized, each bird received 0.3 ml of a heparin solution (1000 IU per ml 0.1 M phosphate buffer; Sigma Chemical Co.) followed by transcardial injection of 0.1 M phosphate buffer and 4% buffered paraformaldehyde. Brains were stored *in situ* in 4% paraformaldehyde at 4°C for 24 hours, then were dissected out, weighed, and stored in a sodium azide-containing buffer solution at 4°C for 4 days, followed by a 30% sucrose solution at 4°C for 4 days. At this time, they were frozen on powdered dry ice and stored at -70°C until further processed. Brains were coronally sectioned (section thickness = 35 μm) on a cryostat, and alternate sections were collected on gelatin-coated slides and stained for Nissl substance using thionin. We used the MCID image analysis system (Imaging Research, St. Catherine, Canada) as described in Gulledge and Deviche (1998) to measure the volumes of four VCRs: HVc, RA, MAN, and Area X. We also measured the volume of a control region not associated with the control of song (nucleus rotundus, Rt). Regions were identified using the canary stereotaxic atlas (Stokes, Leonard, and Nottebohm, 1974; Nottebohm *et al.*, 1976). Lateral and medial MAN were measured together due to the difficulty distinguishing the boundary between them. Volumes of HVc were measured using the inclusive boundaries for the nucleus as described in Kirn, Clower, Kroodsma, and DeVoogd (1989). Telencephalon width was measured to

determine if overall brain sizes differed between groups. To do this, three sections with the anterior commissure present were chosen from each brain. The width of the telencephalon at the widest point on each section was then measured and averaged over the three sections.

All methods were approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks and met the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RESULTS

Comparison of intact, castrated, and T-treated castrated photostimulated males

To determine whether T treatment to castrated photostimulated males induced physiological effects, we compared the STIM-I, STIM-C, and STIM-T groups using one-way and one-way repeated measures Analyses of Variance (ANOVA), followed by Student Newman-Keuls (SNK) pair-wise multiple comparisons tests when appropriate. All data sets except song rates met assumptions of normality and equal variance. Therefore, song data were ranked prior to analysis.

Plasma T levels were higher in STIM-T than in STIM-I or STIM-C birds on both sampling dates ($F_{2,21} = 104.0$, $p = 0.0001$; SNK, $p < 0.05$; Table 2), but they were within the range of those measured at the beginning of the breeding season in free-living males (Deviche, Wingfield, and Sharp, in press). Time and treatment interacted to affect CP width ($F_{2,21} = 13.1$, $p = 0.0002$; Table 2). On Day 12, STIM-T birds had larger CPs than STIM-I birds, who in turn had larger CPs than STIM-C birds. On Day 33, STIM-T and

STIM-I birds had similar CP widths, and they were both larger than CP widths of STIM-C birds.

Song rates were significantly higher on Days 7-10 and 28-31 in STIM-T and STIM-I males than in STIM-C males, which did not sing ($F_{2,21} = 11.65$, $p = 0.0004$; SNK, $p < 0.05$; Table 2). VCR volumes and other brain measures did not differ between STIM-I, STIM-C, and STIM-T groups (Figures 2 and 3).

Comparison of intact and castrated photorefractory males

We compared brain measurements and CP widths of REF-CX and REF-C juncos to test the possibility that methodological differences (time spent in captivity, surgery, etc.) between experiments 1 and 2 resulted in differences between these two groups. Both data sets met assumptions of normality and homoscedasticity for all variables. Therefore, Students t-tests were utilized to compare VCR volumes, brain weight, and telencephalon width between the two groups. CP widths were analyzed using one-way repeated measures ANOVA.

REF-CX and REF-C males did not differ with respect to any parameters (p's all > 0.20; Figures 1 and 2; Table 2) and no bird had detectable plasma T. Thus, castrated and intact photorefractory males did apparently not differ from each other in any respect.

Effects of testosterone treatment across photoperiodic conditions.

To investigate whether the effects of T depended on photoperiodic condition, we compared SENS-C, SENS-T, STIM-C, STIM-T, REF-C, and REF-T groups using two by

three factorial ANOVAs and two-way repeated measures ANOVAs, with implant and photoperiodic condition as the independent variables. Data for Area X were heteroscedastic and were ranked prior to analysis. Song data were also ranked prior to analysis. When appropriate, Student Newman-Keuls pair-wise multiple comparisons tests (SNK) were used to determine specific group differences. The proportions of birds singing within T-treated groups were compared using a two by three contingency table and the χ^2 statistic. Correlations between HVc volume and song rate, and between plasma T and song rate, were made with Spearman rank correlations. Due to unequal variance, gonad weights in REF-C and REF-T birds were compared with the Mann-Whitney U-test.

Control birds had non-detectable plasma T levels, whereas T-treated birds had high plasma T levels that did not differ between the two sampling times (Tables 1 and 2; effects of time and interactions between time and all other ANOVA main factors: p's all > 0.6). One STIM-C bird had detectable T (0.8 ng/ml) on Day 12, and was, therefore, eliminated from the study. SENS-C, STIM-C, and REF-C birds had small CPs that did not differ from each other. CP widths were larger in birds receiving T-filled than empty implants, regardless of photoperiodic condition (Tables 1 and 2). CP widths were similar in all T-treated birds, indicating that photoperiodic condition did not modulate the effects of T-implants on this parameter. In experiment 2, REF-T birds had heavier gonads (128 ± 74 mg) than REF-C birds (20 ± 5 mg; $U_{0.05,9,12} = 92$, $p = 0.02$). Molt progressed normally in REF-C males, but stopped in REF-T males.

No bird receiving empty implants ever sang. All SENS-T and STIM-T males sang, but only 2/3 of the REF-T birds sang. Neither median song rates (Tables 1 and 2; effects of time and all time interactions; $p > 0.12$) nor the proportion of birds singing ($\chi^2_{0.05,2} = 3.6101$, $p > 0.25$) differed between these T-treated groups. Song rates of T-treated birds did not correlate with plasma T levels ($r^2 = 0.052$, $p = 0.79$) or HVc volumes ($r^2 = -0.206$, $p = 0.29$).

Photostimulation of castrated males increased the volume of HVc. This effect was not enhanced by concurrent T administration (comparison of STIM-C and STIM-T males: SNK, $p > 0.05$; Table 1; Figure 2). T treatment was equally effective in increasing HVc volume in photosensitive (STIM-T) and refractory (REF-T) males. RA volumes showed the same pattern of change as HVC, but the six groups of birds did not differ significantly from each other. T-treatment increased MAN volume in photorefractory birds to the same size as that of photosensitive and photostimulated birds. REF-C birds had smaller MAN volumes than all other groups (SNK, $p < 0.05$). Photoperiodic condition influenced Area X volumes (Table 1; Figure 2), but multiple pairwise comparisons tests did not reveal significant differences across groups. T treatment did not affect Area X volume, regardless of photoperiodic condition. Telencephalon width, Rt volume, and brain weight were similar in all groups (Table 1; Figure 3).

Effect of photoperiodic condition in castrated males.

To determine the effect of photoperiodic condition on VCR volume changes in castrated males, we compared these volumes between SENS-C, STIM-C, and REF-CX

groups using one-way ANOVAs, followed by SNK multiple comparisons tests when appropriate. All data sets met assumptions of normality and equal variance.

HVc volumes differed across photoperiodic conditions ($F_{2,21} = 4.13$, $p = 0.03$; Figure 2). The previously noted increase in HVc volume resulting from photostimulation dissipated as birds became photorefractory. All other VCR volumes and brain measures were similar among groups (all $p > 0.20$; Figures 2 and 3).

DISCUSSION

This study was designed to examine the independent and synergistic effects of T and photoperiodic condition on song production and VCR volumes in Dark-eyed Juncos. Our results support previous studies in that we found effects of T as well as photoperiodic condition on VCR volumes. However, we report here for the first time that LD exposure to castrated photosensitive males increases HVc volume maximally. Unlike in White-crowned Sparrows (Smith *et al.*, 1997b), concurrent T administration does not further increase HVc volume in juncos. Also in contrast to other studies, we found that song production in response to T treatment does not depend on photoperiodic condition. In addition, a large HVc is not necessarily associated with song production, as photostimulated castrates never sang despite having large HVc volumes. Finally, we have shown that castrated males show seasonal plasticity in HVc volume in the absence of detectable circulating T.

One purpose of this study was to determine the effects of T treatment on song rates in adult male Dark-eyed Juncos across photoperiodic conditions. T treatment

induced comparable circulating levels of this steroid, irrespective of whether birds were photosensitive and held on short days, photostimulated, or photorefractory. T treatment also induced similar song rates regardless of photoperiodic condition. Song rate was not correlated with HVc volume, which is consistent with a finding by MacDougall-Shackleton, Hulse, and Ball (1998). There were no differences in song rates or in the proportion of birds singing in each photoperiodic condition. Our results differ from those of Nowicki and Ball (1989), who found that photostimulation increased song rate independent of T. However, that study did not include untreated controls. In addition, Nowicki and Ball measured song rates after photostimulated birds had been exposed to T for 6-8 weeks, whereas photosensitive birds had been exposed to T for only 1 to 4 weeks. Therefore, the increase in song rate found in photostimulated birds could have been an effect of a longer total exposure to T instead of an independent effect of photoperiod (refer to Smith *et al.*, 1997b, for a detailed description). We conclude that adult male juncos have the potential to respond behaviorally to T treatment irrespective of their photoperiodic condition. Because we only measured average song rates of birds, additional studies are needed that will investigate whether juncos given exogenous T in different photoperiodic conditions differ with respect to their song structure. Smith *et al.* (1997a) found differences in song attributes across seasons in adult male Song Sparrows. Specifically, trill length, note structure stereotypy, and the rate of song type variations changed seasonally. However, changes in these attributes all coincided with changes in T levels, indicating that photoperiodic condition does not play a major role.

Our second purpose was to determine the relative importance of T treatment and photoperiodic condition on the control of VCR volumes and to determine relationships between VCR volumes and singing behavior. Song production was associated with a large HVc in all photoperiodic conditions. This result does not necessarily indicate a causal relationship between HVc volume and song, and in fact we found no correlation between HVc volume and song rate. T administration to SD photosensitive or to photorefractory birds increased HVc volume to the same degree, and HVc size did not differ among T-treated birds, regardless of photoperiodic condition. Therefore, adult male Dark-eyed Juncos can increase their HVc volumes to the same size whether they receive T treatment while photosensitive or photorefractory. However, exposure to LD was sufficient to increase HVc volume in photostimulated birds, and concurrent T treatment did not increase this volume further. These results differ from those of Smith *et al.* (1997b). These authors found an increase in HVc volume induced by LD exposure, but also reported that T treatment induced an additional volume increase in photostimulated adult male Gambel's White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*). Our results also differ from those of Bernard *et al.* (1997) who found that T administration to photorefractory male European Starlings does not increase HVc volume to a size similar to that of T-treated SD photosensitive males. However, this study did not include a photorefractory group that did not receive T implants, so we do not know if T treatment had even a small effect on HVc in photorefractory birds.

All photostimulated castrated juncos had large HVc volumes, but only males that received T sang, indicating that this steroid is required for song expression. Further, the

volumes of HVc in castrated photostimulated and T-treated castrated photosensitive birds did not differ. Thus, LD exposure may increase HVc volume, but T alone could do it to the same degree. It is likely that photoperiod and T exert different cellular effects on the HVc and possibly RA. Smith *et al.* (1997b) showed that photoperiod and T interact to increase the cross-sectional area of neurons in the HVc in Gambel's White-crowned Sparrows, whereas T alone increases the number of neurons in HVc. Together, they both cause an increase in HVc volume. Juncos that breed in Interior Alaska are naturally exposed to LD starting in March, i.e., over one month before reaching their breeding grounds. In other migratory species, plasma T levels remain relatively low in males until they reach their breeding areas (Wingfield and Farner, 1978a; 1978b). We suggest that increasing HVc volume in response to LD, but before high plasma levels of T are reached, may facilitate song production as soon as birds arrive on their breeding territories. This would be particularly adaptive in situations where the period that is favorable for completing breeding activities is very brief, as is generally the case at high latitudes. Different cellular effects of T and photoperiod could also help explain why LD castrates do not sing even though they have large HVc volumes. Additionally, song production may require stimulating effects of T on the syrinx musculature (Deviche and Schumacher, 1982; Luine, Nottebohm, Harding, and McEwen, 1980).

The mechanism of action of the T-independent changes in VCR volumes is, as of yet, unknown. A recent investigation reported effects of melatonin on VCR volumes (Bentley, Van't Hof, and Ball, 1999). Specifically, exogenous melatonin treatment to male European Starlings attenuated the LD-induced increase in HVc volumes and

decreased the volume of Area X. Other studies have described melatonin binding sites within the song system (Gahr and Kosar, 1996; Whitfield-Rucker and Cassone, 1996). Thus, melatonin is a potential mediator of T-independent LD-induced volume changes.

The third goal of this study was to determine if the VCRs of castrated birds undergo seasonal changes in volume. To our knowledge, this is the first study investigating seasonal VCR volume plasticity in males that were castrated *prior to* photoperiodic manipulations. For example, Bernard *et al.* (1997) found testis-dependent and -independent effects of photoperiod in American Tree Sparrows, but they castrated photorefractory sparrows *after* they had become photorefractory. Bentley *et al.* (1999) also compared VCR volumes of castrated photorefractory starlings with those of photostimulated and photosensitive birds. However, starlings in that study were also castrated when photorefractory, and the authors did not report whether their photosensitive birds were castrated. Therefore, prior to the present work, it had not been determined whether birds castrated while on SD (i.e., prior to the breeding season) would undergo seasonal changes in VCR volumes as they became photostimulated and then photorefractory. We found that castrated photorefractory birds had smaller HVc volumes than castrated photostimulated birds. This suggests that the seasonal changes in HVc volume in male Dark-eyed Juncos can be mediated entirely by photoperiodic condition in the absence of gonadal steroids.

In the present work, Area X and MAN were large in both control and T-treated SD photosensitive castrated birds. Area X in adolescent male juncos is the same size as in breeding adult males, even though plasma T levels are low in adolescence and high

during the breeding season (Gulledge and Deviche 1997). Taken together, these results indicate that Area X volume increases in an androgen-independent fashion in adolescents in the fall, and is still large in early spring, when birds are still exposed to SD and have non-detectable T levels. Gulledge and Deviche (1997) found that castrating adult male juncos during the breeding season caused Area X to shrink compared to T-treated castrated birds. This indicated that T was necessary to maintain large Area X volumes during the breeding season. Although we found no difference between T-treated and control castrated photostimulated males, we saw a trend in photorefractory birds for T treatment to increase Area X volume, supporting the idea that Area X volume becomes androgen-dependent *after* a bird's first breeding season.

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Legend to Figures

Figure 1. Design of experiment 1. Abbreviations: SD, short days; LD, long days; n, sample size; SENS-C, photosensitive control; SENS-T, photosensitive T-treated; STIM-C, photostimulated control; STIM-T, photostimulated T-treated; STIM-I, photostimulated intact; REF-CX, photorefractory castrated.

Figure 2. Volumes of Area X, RA, HVc, and RA of photosensitive (SENS), photostimulated (STIM), and photorefractory (REF) adult male juncos 35 days after receiving T-filled or empty implants. Also included are intact photostimulated (STIM-I) and castrated photorefractory (REF-CX) males. Data are presented as means ± standard deviations, except for Area X data, which are presented as medians ± 0.05 interquartile intervals. Different letters indicate significant differences among groups analyzed with two-way ANOVAs (Student Newman-Keuls, $p < 0.05$).

Figure 3. Rt volume, telencephalon width, and brain weight of photosensitive (SENS), photostimulated (STIM), and photorefractory (REF) adult male juncos 35 days after receiving T-filled or empty implants. Also included are intact photostimulated (STIM-I) and castrated photorefractory (REF-CX) males. Data are presented as means ± standard deviations.

Figure 1.

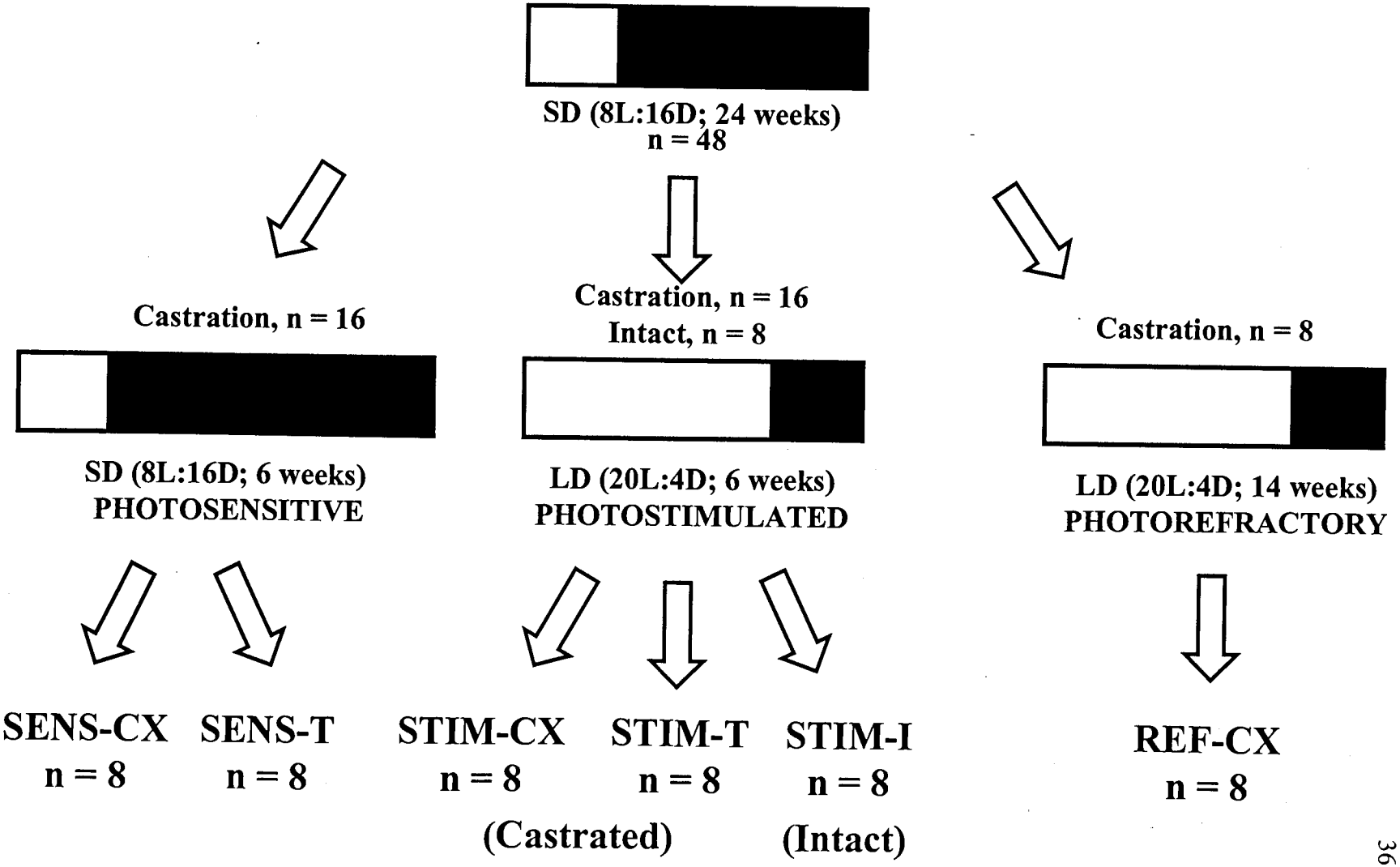
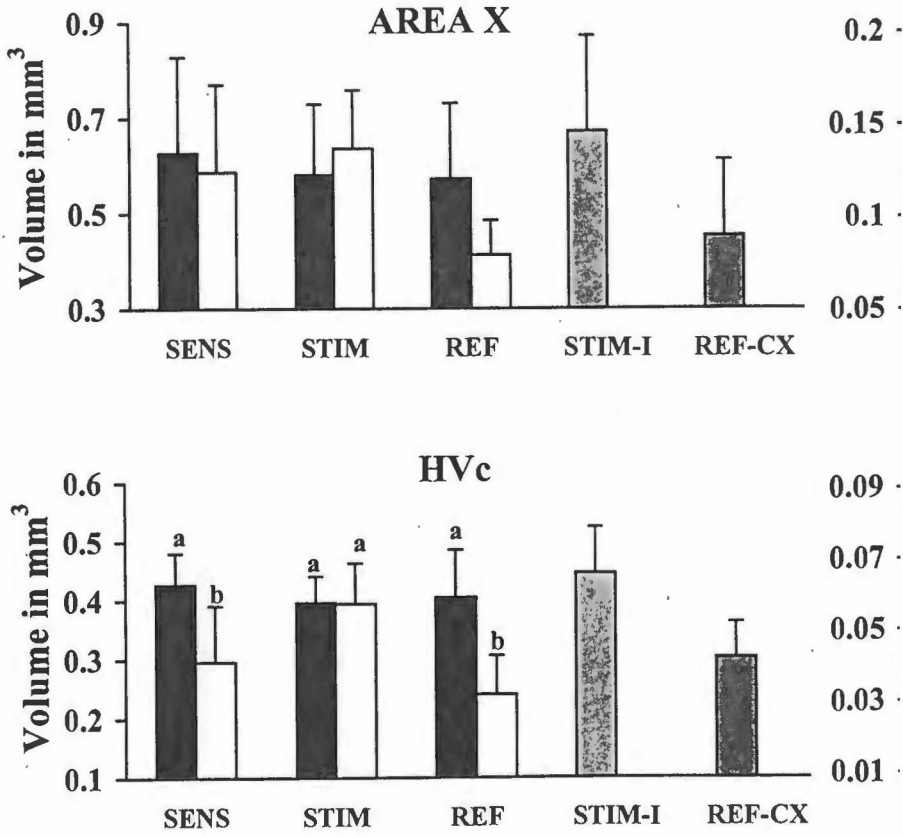
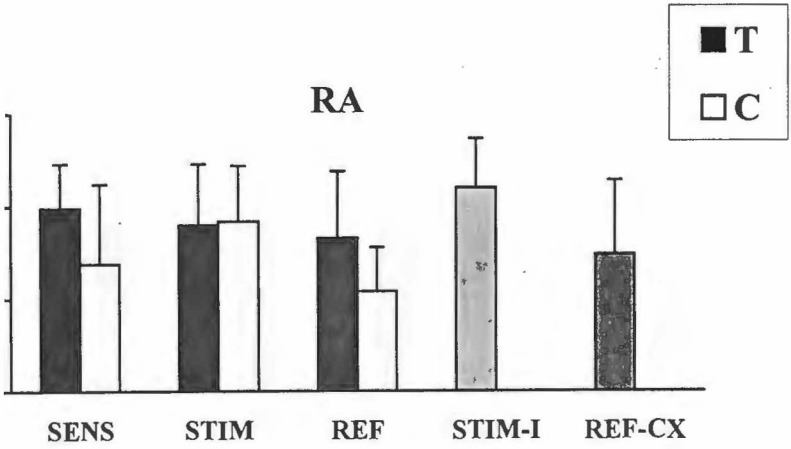

SD (8L:16D; 24 weeks)
n = 48
Castration, n = 16
Castration, n = 16
Intact, n = 8
Castration, n = 8
SD (8L:16D; 6 weeks)
PHOTOSENSITIVE
LD (20L:4D; 6 weeks)
PHOTOSTIMULATED
LD (20L:4D; 14 weeks)
PHOTOREFRACTORY
SENS-CX
n = 8
SENS-T
n = 8
STIM-CX
n = 8
STIM-T
n = 8
STIM-I
n = 8
(Castrated)
(Intact)
REF-CX
n = 8

Figure 2.


AREA X
Volume in mm3
0.9
0.7
0.5
0.3
0.2
0.15
0.1
0.05
SENS
STIM
REF
STIM-I
REF-CX
HVc
Volume in mm3
0.6
0.5
0.4
0.3
0.2
0.1
0.09
0.07
0.05
0.03
0.01
a
b
a
a
a
b
SENS
STIM
REF
STIM-I
REF-CX


T
C
RA
SENS
STIM
REF
STIM-I
REF-CX

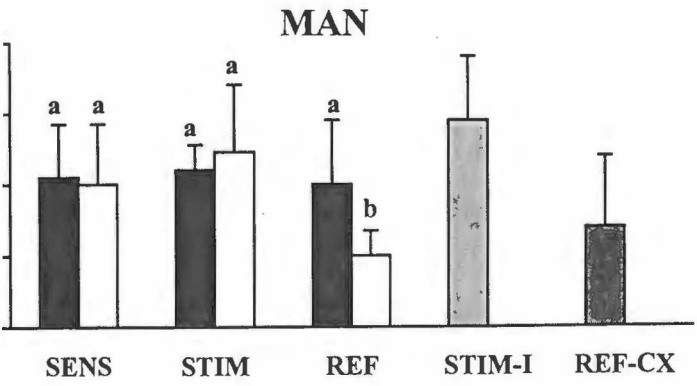
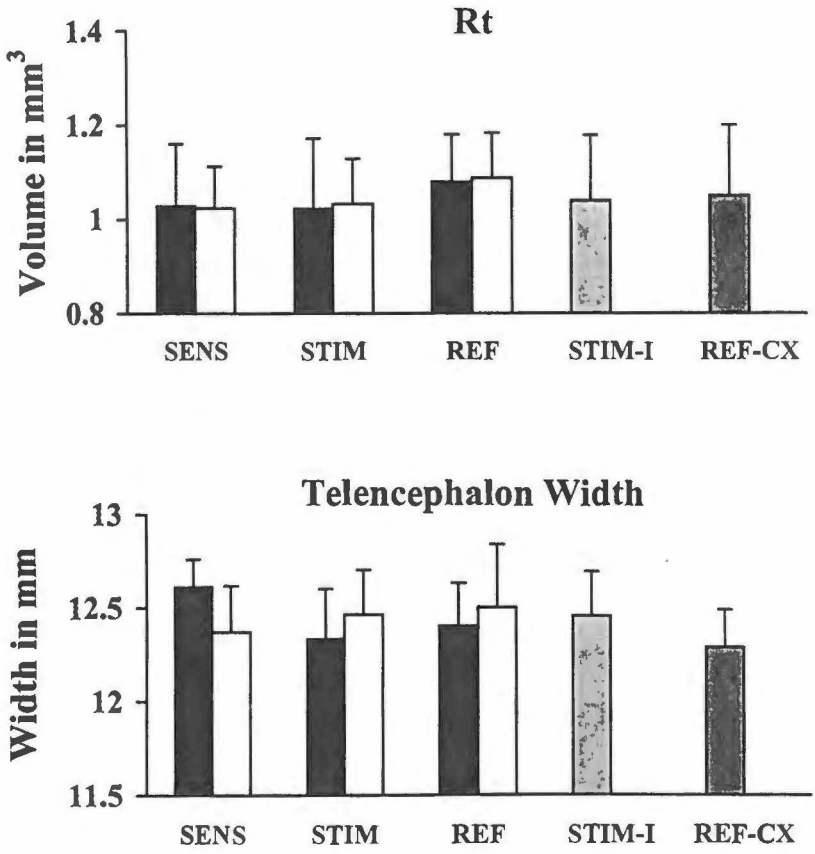

MAN
a
a
a
a
a
b
SENS
STIM
REF
STIM-I
REF-CX

Figure 3.


Rt
Volume in mm3
1.4
1.2
1
0.8
SENS
STIM
REF
STIM-I
REF-CX
Telencephalon Width
Width in mm
13
12.5
12
11.5
SENS
STIM
REF
STIM-I
REF-CX

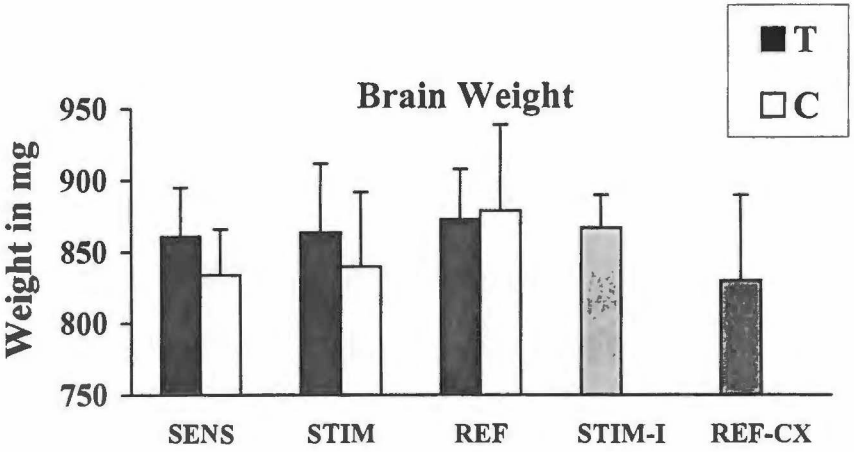

Brain Weight
T
C
Weight in mg
950
900
850
800
750
SENS
STIM
REF
STIM-I
REF-CX

Table 1. Two-way ANOVA results for analysis 2.

Parameter	Photoperiodic Condition	Testosterone	P x T[1]
Area X volume	$F_{2,46} = 3.81, p = 0.03$	$F_{1,46} = 1.28, p = 0.26$	$F_{2,46} = 2.77, p = 0.07$
RA volume	$F_{2,46} = 1.34, p = 0.27$	$F_{1,46} = 3.44, p = 0.07$	$F_{2,46} = 2.81, p = 0.07$
HVc volume	$F_{2,46} = 4.55, p = 0.02$	$F_{1,46} = 24.6, p < 0.001$	$F_{2,46} = 6.07, p < 0.001$
MAN volume	$F_{2,46} = 6.81, p < 0.001$	$F_{1,46} = 2.72, p = 0.11$	$F_{2,46} = 4.53, p = 0.02$
Rt volume	$F_{2,46} = 0, p = 0.96$	$F_{1,46} = 1.51, p = 0.23$	$F_{2,46} = 0.01, p = 0.99$
Brain weight	$F_{2,46} = 1.13, p = 0.32$	$F_{1,46} = 0.05, p = 0.89$	$F_{2,46} = 0.07, p = 0.78$
Tel. width	$F_{2,46} = 3.81, p = 0.03$	$F_{1,46} = 3.81, p = 0.03$	$F_{2,46} = 3.81, p = 0.03$
CP width[2]	$F_{2,77} = 2.57, p = 0.08$	$F_{1,77} = 196, p < 0.0001$	$F_{2,77} = 0.42, p = 0.66$
Plasma T[2]	$F_{2,77} = 2.33, p = 0.13$	$F_{1,77} = 551, p < 0.0001$	$F_{2,77} = 1.65, p = 0.20$
Song rate[2]	$F_{2,77} = 0.34, p = 0.71$	$F_{1,77} = 56.3, p < 0.0001$	$F_{2,77} = 0.08, p = 0.91$

[1] P x T = photoperiodic condition x testosterone interaction
[2] CP widths, plasma T levels, and song rates were analyzed using two-way repeated measures ANOVA. Significant effects of time or time interactions are presented in the text.

Table 2. Plasma T levels, CP width, and song rates.

	Plasma T (ng/ml)[1,2]		CP Width (mm)[1,2]		Song Rate[2,3]	
Photoperiodic condition	Day 12	Day 33	Day 12	Day 33	Days 7-10	Days 28-31
SENS-T (n = 8)	11.50 ± 2.90[a]	12.19 ± 3.74[a]	4.6 ± 0.6[a]	4.7 ± 0.6[a]	24 ± 20[a]	15 ± 17[a]
SENS-C (n= 8)	n.d.[b]	n.d.[b]	3.6 ± 0.4[b]	3.4 ± 0.4[b]	0[b]	0[b]
STIM-T (n = 8)	13.95 ± 2.90[a]	13.97 ± 5.72[a]	5.0 ± 0.6[a]	5.1 ± 0.4[a]	21 ± 19[a]	18 ± 18[a]
STIM-C (n = 7)	n.d.[b]	n.d.[b]	3.7 ± 0.3[b]	3.7 ± 0.3[b]	0[b]	0[b]
REF-T (n = 12)	12.44 ± 3.29[a]	12.35 ± 4.92[a]	4.6 ± 0.5[a]	4.8 ± 0.6[a]	16 ± 19[a]	24 ± 23
REF-C (n = 10)	n.d.[b]	n.d.[b]	3.7 ± 0.3[b]	3.5 ± 0.3[b]	0[b]	0[b]
STIM-I (n=8)	1.06 ± 1.09	0.62 ± 0.66	4.0 ± 0.4	5.6 ± 0.8	12 ± 9	13 ± 17

[1] Means ± standard deviations.
[2] Days indicate the number of days after the onset of testosterone treatment.
[3] Median number of songs counted during 30 min observation periods ± 0.5 interquartile intervals.
n.d. = non-detectable
[a,b] Different letters indicate significant differences among groups.

III. CHRONIC OPIOID RECEPTOR BLOCKADE DOES NOT AFFECT SONG PRODUCTION OR VOCAL CONTROL REGION VOLUMES IN ADULT MALE DARK-EYED JUNCOS (*Junco hyemalis*)

(As submitted to *Hormones and Behavior* as Dloniak and Deviche)

ABSTRACT

Photoperiodic adult male songbirds display pronounced seasonal variation in song production and vocal control region (VCR) volumes that correlate with changes in plasma testosterone (T) levels. VCRs contain opioid peptides and receptors, but the functions of these peptides and receptors in the vocal control system are unknown. This study investigated the effects of chronic opioid receptor blockade induced by the administration of a selective opioid receptor antagonist, naloxone, on song production, VCR volumes, and reproductive physiology of adult male Dark-eyed Juncos (*Junco hyemalis*) exposed either to short or to long days. Birds exposed to long days sang and had larger HVc and Area X volumes, as well as larger gonads and higher plasma testosterone (T) levels, than did birds exposed to short days, which never sang. Naloxone treatment did not influence song production, VCR volumes, gonad weight, or plasma T concentrations regardless of photoperiod, suggesting that opioids are not involved in regulating adult song production or VCR volume plasticity. Although apparently not involved in the regulation of song expression, VCR opioids and their receptors may control other aspects of vocal behavior such as song learning or auditory processing.

INTRODUCTION

In songbirds, song learning and production are controlled by an interconnected set of brain regions (vocal control regions, VCRs) collectively called the song system (Nottebohm, Stokes, and Leonard, 1976; Vicario, 1991; Johnson and Bottjer, 1992). In species that breed seasonally, the song system exhibits neuronal plasticity. The volumes of several VCRs, as well as the size, density, and replacement rate of neurons within certain VCRs, change across seasons (Brenowitz, Nalls, Wingfield, and Kroodsma, 1991; Nottebohm, 1981; Alvarez-Buylla and Kirn, 1997). For example, in male Dark-eyed Juncos (*Junco hyemalis*, a sexually dimorphic, seasonal oscine), the volumes of two VCRs (Area X of the parolfactory lobe and HVc, the high vocal center) are larger during than after the breeding season (Gulledge and Deviche, 1997).

Seasonal changes in plasma testosterone (T) levels correlate to changes in VCR volumes. When seasonally breeding male birds experience a long photoperiod (> approx. 12 hours of light per day), the reproductive system is stimulated. Plasma T levels rise, gonad size increases, and birds start to sing (Wingfield and Hahn, 1994; Smith, Brenowitz, Wingfield, and Beecher, 1997; Deviche, Wingfield, and Sharp, in press). During the breeding season, VCR volumes are also large (Nottebohm, 1981; Kirn, Clower, Kroodsma, and DeVoogd, 1989; Bernard and Ball, 1995). After the breeding season, birds have very low to non-detectable T levels, gonads regress, VCRs decrease in size, and singing decreases or stops (Smith, 1996; Brenowitz, Baptista, Lent, and Wingfield, 1996; Gulledge and Deviche, 1997). The seasonal pattern of changes in VCR volumes can be simulated in captive birds by exposure to long photoperiod or

administration of T (Nottebohm, 1981; Brenowitz, Nalls, Wingfield, and Kroodsma, 1991; Smith, Brenowitz, Wingfield, and Baptista, 1995). The effects of T may be mediated by androgen receptors located in the VCRs (HVc, the robust nucleus of the archistriatum (RA), and the magnocellular nucleus of the anterior neostriatum (MAN); Smith, Brenowitz, and Prins, 1996; Bottjer, Meismer, and Arnold, 1984; Arnold, 1980). Although Area X does not have androgen receptors, it receives projections from HVc, and the effects of T on Area X may be mediated by these projections (Arnold, 1980; Gahr, 1990).

The VCRs of several species contain opioid peptide-like immunoreactivity (Ryan, Arnold, and Elde, 1981; Ball, Faris, Hartman, and Wingfield, 1988; Ball, Richardson, and Balthazart, 1995; Bottjer and Alexander, 1995; Deviche and Gunturkun, 1992; Carrillo and Doupe, 1995). In addition, VCRs of Dark-eyed Juncos contain receptors that are specific for these peptides (Gulledge and Deviche, 1995, 1999), suggesting that opioid peptides bind to these receptors within the VCRs. The functions of opioid peptides and receptors in the song system are, however, unknown.

In other vertebrates, including non-oscine birds, opioid peptides and their receptors regulate many types of behavior, including sexual behavior and vocalizations. For example, administration of opioid agonists disrupts male sexual behavior in rats (Matuszewich, Ormsby, Moses, and Lorrain, 1995; Bitran and Hall, 1987; McIntosh, Vallano, and Barfield, 1980), while opioid antagonists facilitate it (Myers and Baum, 1979; McIntosh *et al.*, 1980). In socially isolated chicks, opioid peptides reduce distress vocalizations (Panksepp, Vilberg, Bean, Coy, and Kastin, 1978a), whereas opioid

receptor blockade with the selective opioid antagonist naloxone increases these vocalizations (Panksepp, Bean, Bishop, Vilberg, and Sahley, 1980; Sufka, Hughes, McCormick, and Borland, 1994). These results suggest a role for VCR opioid peptides in the control of vocal expression. Gulledge and Deviche (1995), however, found no seasonal differences in δ, κ, or μ opioid receptor densities between breeding (singing) and post-breeding (non-singing) adult male juncos, suggesting that seasonal differences in song production and VCR volumes do not result from modifications in VCR opioid receptor densities. Although *receptor* densities do not change seasonally, there could be behaviorally important changes in opioid *peptide* synthesis and secretion, resulting in seasonal changes in receptor occupancy. Therefore, the *first* purpose of this study was to determine whether blockade of opioid receptors by naloxone influences song expression in adult male juncos.

Gonadal steroids influence VCR plasticity (Nottebohm *et al.*, 1987; Smith *et al.*, 1995; Gulledge and Deviche, 1997). The mechanisms involved in this influence remain largely speculative, although recent work has indicated that some of the effects of testosterone on neuronal recruitment are mediated through brain-derived neurotrophic factor (BDNF) (Rasika, Alvarez-Buylla, and Nottebohm, 1999). These steroids influence the opioid system (Morrell, Schwanzel-Fukuda, Fahrbach, and Pfaff, 1984; Wardlaw, Thoren, and Franz, 1982; Bhanot and Wilkinson, 1983; Van Vugt, Sylvester, Aylsworth, and Meites, 1982), and opioids themselves exert widespread effects on cell survival and plasticity. Specifically, these peptides can inhibit neural cell proliferation (Zagon and McLaughlin, 1987; Zagon and McLaughlin, 1991) and increase neural cell survival

(Sakaguchi, Fugimori, Satoh, Satoh, Takeuchi, and Matsumura, 1999; Meriney, Ford, Olivia, and Pilar, 1991). Therefore, opioids may meditate some effects of steroids on the VCRs, and our *second* purpose was to examine the effects of naloxone treatment on VCR volume plasticity.

Elevated T levels are required for song expression in juncos (see chapter II). Naloxone increases circulatory T levels in rabbits (Pedron, Gonzalez-Unzaga, and De Celis, 1998) and suppresses testicular growth at certain times in the Red Munia, *Estrildo amandava* (Lal and Rajeshwari Devi, 1997). Thus, any effects of naloxone administration on the vocal control system could be mediated by alterations of gonadal function. To test this hypothesis, we determined the effects of naloxone treatment on gonad weight and plasma T levels.

Birds exposed to short days have an inactive hypothalamalo-pituitary-gonadal axis (low plasma T, no singing), whereas birds exposed to long days have an active axis (high plasma T, singing; Nicholls *et al.*, 1988). Therefore, we administered naloxone to both short day and long day birds in order to determine whether the effects, if any, of the antagonist were stimulatory (i.e., stimulating song production or increasing VCR volumes in short day birds) or inhibitory (i.e., decreasing or eliminating song production or decreasing VCR volumes in long day birds). Because administration of naloxone causes short-term anorexia in birds (Deviche 1992; Deviche, Melmer, and Schepers, 1984; Deviche and Schepers, 1984), we measured food consumption, feeding behavior, and body condition. Finally, we measured several other behaviors (preening, drinking, and locomotion) to determine the behavioral specificity of the effects resulting from

opioid receptor blockade.

MATERIALS AND METHODS

Animal Collection and Housing

Thirty-two adult male Dark-eyed Juncos were collected from a wild population near Fairbanks, Alaska (65°N, 148°W), between May 1 and 15, 1997, i.e., at the beginning of the breeding season. At this time, males sing at high rates, plasma T levels are elevated, and the reproductive system is rapidly recrudescing (Deviche, Wingfield, and Sharp, in press). Birds were lured into mist nets by playbacks of taped conspecific song. All birds were brought to the University of Alaska Fairbanks and housed in individual cages, with Mazuri parrot and small bird pelleted food (PMI Nutrition Int., St. Louis, MO) and Avi-Con vitamin treated-water (Vet-A-Mix Inc., Shenandoah, IA) available *ad libitum* throughout the experiment. Birds were not visually or acoustically separated from one another and were initially exposed to short days (SD; 7L:17D; lights on at 0800 hrs) to slow gonadal recrudescence.

Experimental Design

On May 23 (Day 1), birds were randomly divided into two groups. Sixteen males remained exposed to SD, and the rest (n=16) were transferred to long days (LD; 20L:4D; lights on at 0800 hrs). At this time, 8 SD and 8 LD males (SD-NAL and LD-NAL groups) each received an i.p. Alzet minipump (model 1002; capacity 0.1 ml; Alza Pharmaceuticals, Palo Alto, CA) prefilled with a solution of naloxone hydrochloride

(129.5 mg/ml sterile saline; Endo Pharmaceuticals, New York). Given a putative release rate of 0.3089 μl/hr (figure provided by Alza Pharmaceuticals), each bird (mean body mass: 20 g) received on average 2 mg/kg body mass/hr of naloxone. There are no published avian studies using naloxone-containing Alzet minipumps. Therefore, the above dosage was used based on mammalian investigations in which similar or smaller dosages induced physiological and/or behavioral effects (Jaloweic, Panksepp, Zolovick, Najam, and Herman, 1981; Weisenfeld and Hallin, 1983; Rocha, Ackermann, Nassir, Chugani, and Engel, 1993; Alcaraz, Vargas, Fuente, and Milanes, 1993; Malin, Hemple, Exley, and Addington, 1986). The remaining birds (SD-CON and LD-CON) each received a saline-filled pump. Pumps were administered under complete anesthesia induced by an intramuscular injection of a xylazine/ketamine solution (0.032 mg xylazine and 1.6 mg ketamine per 0.2 ml sterile saline; ketamine from Phoenix Pharmaceutical Inc., St. Joseph, MO, and xylazine from Loyd Laboratories, Shenandoah, IA). On June 4 (Day 13) all pumps were removed and replaced with identically prepared pumps. Pumps were opened and inspected when removed to verify naloxone release. Birds were killed by perfusion on June 16 (Day 25). All methods were approved by the Animal Care and Use Committee of the University of Alaska Fairbanks and met the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood Collection and Testosterone Assay

All birds were bled from an alar vein on May 22, June 3, and June 15 (Days 0, 12,

and 24). After centrifugation, the plasma was drawn off and stored at -20° C until assay. Plasma samples (25µl) were assayed for total testosterone (T) by radioimmunoassay using a commercial kit (Diagnostic Products Corporation, Los Angeles, CA). This assay has been used previously for measuring T in juncos (see Gulledge and Deviche, 1998, for further details) and is both sensitive (lower detection limit: 10 pg/assay tube) and specific (cross-reactivity: 3.3% with dihydrotestosterone, 0.02% with estradiol). All samples were assayed in duplicate in a single series. The intraassay coefficient of variation was 4.6%.

Morphology

We measured the body mass, cloacal protuberance (CP) width, and furcular fat score of each bird on days 12 and 24. Body mass was measured to the nearest 0.1 g. CP width (an index of gonadal development: Schwabl and Farner, 1989; Deviche, 1992) was measured to the nearest 0.1 mm. Furcular fat scores vary between 0 (empty furcular space) and 5 (furcular space filled with fat) and correlate with total body fat (Rogers, 1991; Silverin, Viebke, and Westin, 1989).

24-hour food consumption

We determined the 24-hour food intake of each bird to the nearest 0.1 gram at two times during the experiment: 8 to 10 and 16 to 18 days after implantation. Food intake was defined as the mean difference between the weight of food in feed dishes at the beginning and end of a 24-hour period over three consecutive days.

Behavior

Each bird was videotaped for one hour while in its home cage on two different days between June 10 and 13, for a total of two hours of observation for each bird. Videotaping was done in a random order and between 0900 hours and 1330 hours to minimize the influence of daily fluctuations in behavior frequencies. Each tape was subsequently watched by two independent observers who were unaware of the experimental treatment received by each bird. The number of songs produced by each bird was counted, and the occurrence (presence/absence during each minute of observation) of specific behaviors (singing, preening, feeding, locomotion, drinking) was determined.

Tissue processing and VCR volume measurement

All birds were killed by *in vivo* perfusion. For this, they were first anesthetized by a xylazine/ketamine pectoral injection, followed by methoxyflurane inhalation (Metofane: Pitman-Moore Inc., Mundelerin, IL). Birds then received 0.3 ml of a heparin solution (1000 IU per 0.1 M phosphate buffer; Sigma Chemical Co., St. Louis, MO) intracardiacally, followed by transcardial injection of 0.1 M phosphate buffer and 4% buffered paraformaldehyde. Testes were removed and weighed at the time of perfusion. Brains were stored *in situ* in 4% paraformaldehyde at 4°C for 24 hours, then were dissected out, weighed, and stored in sodium azide-containing buffer at 4°C for 4 days, followed by 30% sucrose-containing buffer at 4°C for 4 days. They were then frozen on

powdered dry ice and stored at -70°C until processed. Brains were coronally sectioned (section thickness = 35 μm) on a cryostat. Alternate sections were collected on gelatin-coated slides and stained for Nissl substance using thionin.

We used the MCID image analysis system (Imaging Research, St. Catherine, Canada) as described in Gulledge and Deviche (1998) to measure the volumes of four VCRs: HVc, RA, MAN, and Area X. We also measured the volume of a control region, nucleus rotundus (Rt), which is not involved in vocal behavior. Regions were identified using the canary stereotaxic atlas (Stokes, Leonard, and Nottebohm, 1974; Nottebohm *et al.*, 1976). Lateral and medial MAN were measured together due to the difficulty of distinguishing the boundary between them. Volumes of HVc were measured using the inclusive boundaries of the nucleus as described in Kirn, Clower, Kroodsma, and DeVoogd (1989). Telencephalon width was measured as an index of overall brain size. To do this, three sections with the anterior commissure present were chosen from each brain. The width of the telencephalon at the widest point on each section was then measured and averaged over the three sections.

Statistical Analyses

All brain and behavior data, as well as gonad weights, were analyzed using two way analyses of variance (ANOVAs), with naloxone treatment and photoperiod as the independent variables. Data sets for singing and gonad weight did not meet assumptions of homoscedasticity and/or normality and were, therefore, ranked prior to analysis. Morphological data (CP width, body mass, and fat scores), 24-hour food consumption,

and plasma T levels were analyzed using two way repeated measures ANOVAs. When appropriate, multiple pair-wise comparisons were performed using Student Newman Keuls (SNK) tests. Data are expressed as means ± standard deviations or, when transformed to ranks before analysis, as medians ± 0.5 interquartile intervals (Nicholson, Bowyer, and Kie, 1997).

RESULTS

Plasma T levels and reproductive morphology

Exposure to LD stimulated T secretion and reproductive system development. Birds exposed to LD had significantly higher plasma T levels than birds exposed to SD (Figure 1). Photoperiod and time interacted to affect plasma T levels ($F_{2,26} = 9.01$, $p = 0.0033$). These levels did not differ between any of the groups on Day 0. On Days 12 and 24, birds exposed to LD had higher T levels than birds exposed to SD. When sacrificed, LD-exposed birds had developed gonads (LD-CON: 350 ± 59 mg; LD-NAL: 280 ± 100 mg), whereas SD birds had completely regressed gonads (SD-CON: 4.9 ± 1.3 mg; SD-NAL: 4.5 ± 1.5 mg; $F_{3,28} = 89.18$, $p = 0.0001$). LD-exposed juncos also had larger CP widths than SD-exposed juncos ($F_{2,28} = 112.10$, $p = 0.0001$; Table 1). CP widths did not differ between Days 12 and 24. There was no effect of naloxone treatment on plasma T levels, paired testis weights, or CP widths.

Food consumption, feeding frequency, and body condition

Average 24-hour food consumption was higher (Table 2), whereas the number of

visits to the feeder was lower (Table 3), in birds exposed to LD than to SD. Naloxone administration did not affect either 24-hour food consumption or feeding frequency. Photoperiod had no effect on fat reserves, but naloxone and time interacted to affect these reserves (Table 1). The fat scores of naloxone-treated birds increased over time, but a multiple comparisons test revealed no significant differences among groups. There was a significant time x photoperiod interaction effect on body mass. SD birds had greater body mass than LD birds on Day 24 (Table 1). There was no effect of naloxone on body mass.

Song and other behaviors

LD-exposure induced singing (Table 3). No SD-exposed males ever sang, and song rates did not differ between naloxone-treated and control birds on LD. Neither naloxone treatment nor photoperiod affected preening, drinking, or locomotion (Table 3).

Vocal control region volumes

Volumes of HVc and Area X were significantly larger in LD- than SD-exposed males (Figure 2), but were not affected by naloxone treatment. The volumes of RA, MAN, and Rt, as well as brain weight and telencephalon width, did not differ between groups (Figures 2 and 3).

DISCUSSION

Previous studies demonstrated the presence of opioid peptides and receptors in the oscine vocal control system, but the functions of these peptides and receptors in this

system are unknown. In mammals as well as non-oscine birds, vocal behavior is affected by manipulations of the opioid system (dogs: Panksepp, Herman, Conner, Bishop, and Scott, 1978b; Rhesus monkeys: Kalin and Shelton, 1989; rats: Borszcz, Johnson, and Thorp, 1996; chicks: Panksepp *et al.*, 1978a, 1980; Sufka *et al.*, 1994). Other studies have shown that the central opioid system controls aspects of reproductive behavior in mammals (Fabbri, Jannini, Gnessi, Ulisse, Moretti, and Isadori, 1989; Sirinathsinghji, 1986; Weisner and Moss, 1986) as well as oscines (Maney and Wingfield, 1998). Specifically, the latter authors reported that endogenous opioids increase corticotrophin-releasing factor-induced suppression of courtship behavior in female White-crowned Sparrows (*Zonotrichia leucophrys*).

In a previous study, breeding (singing) and post-breeding (non-singing) adult male Dark-eyed Juncos had similar opioid receptor densities in their VCRs (Gulledge and Deviche, 1995). Although opioid *receptor* densities did not differ between singing and non-singing males, seasonal changes in *peptide* production or release may modulate singing behavior. One goal of the present investigation was to investigate this hypothesis by blocking access of endogenous peptides to their receptors. By administering an opioid antagonist to LD- and SD-exposed adult male juncos, we could determine whether chronic opioid receptor blockade inhibits or stimulates singing and/or influences VCR plasticity. Birds exposed to LD had higher plasma T levels and larger CPs than SD-exposed males. LD males also had larger HVc and Area X than SD birds. MAN and RA volumes showed the same trend, but did not differ significantly across groups. Volume differences in HVc and Area X were specific to the song system, as neither telencephalon

width nor Rt volume differed across groups. Birds exposed to LD also sang, whereas birds exposed to SD never sang during the course of the experiment. These data are consistent with those reported in other passerine species (Nottebohm, 1981; Smith, Brenowitz, Beecher, and Wingfield, 1997; Brenowitz *et al.*, 1991) and probably resulted from differences in plasma T levels (Gulledge and Deviche, 1997), although we cannot exclude a T-independent effect of photoperiod (Dloniak and Deviche, in preparation). Within the two photoperiods, however, naloxone and control groups did not differ with respect to VCR volumes or song production. There was also no effect of naloxone administration on any other parameters measured. Previous studies found that acute, as well as chronic, naloxone administration exerts an anorexic effect (rats: Jaloweic *et al.*, 1981; Brands, Thornhill, Hirst, and Gowdy, 1979; juncos: Deviche, 1992; pigeons: Deviche *et al.*, 1984; Deviche and Schepers, 1984). In pigeons, intracranial injection of β-endorphin dose-relatedly increases food consumption (Deviche and Shepers, 1983). In the present study, however, we observed no significant effect of chronic naloxone treatment on feeding frequency, food intake, body mass, or fat reserves. Three mutually non-exclusive hypotheses may explain the absence of effects of naloxone treatment in this study.

First, VCR opioid receptors may not influence song production or adult VCR plasticity in oscines. This hypothesis is consistent with the results of Gulledge and Deviche (1995), which indicated that opioid receptor densities in the VCRs do not differ between breeding and post-breeding adult male juncos.

Second, opioids may be involved in aspects of song behavior other than

expression, such as learning or auditory processing. Gulledge and Deviche (1999) found that, in juncos, VCR opioid receptor densities differ as a function of age and sex. Specifically, adolescent males and females had higher δ and/or μ opioid receptor densities than adults in Area X, RA, and nucleus intercollicularis. This suggests that the opioid system is involved in aspects of vocal control that change with age, such as song learning and VCR development. Opioids have previously been implicated in vocal control system development (Carillo and Doupe, 1995). These authors found that opioid-like immunoreactivity in Area X and RA, but not HVc or MAN, decreased between day 35 and adulthood in male Zebra Finches (*Taeniopygia guttata*), suggesting a role for opioids in the development of these regions. Opioids also facilitate learning in chicks (Schulteis, Janak, Derrick, and Martinez, 1990; Csillag, Stewart, Szekely, Magloczky, Bourne, and Steele, 1993; Columbo, Rivera Martinez, Bennett, and Rosenweig, 1997) and rodents (Rigter, Jansen, Martinez, Messing, Vasquez, Liang, and McGaugh, 1980; Castellano and Pavone, 1985).

Opioids may modulate the filtering of auditory information as it is processed in the VCRs. Opioid receptors are present throughout Area X, HVc, n. intercollicularis, and the songbird auditory cortex analogue (Gulledge and Deviche, 1999), which are all involved in receiving and responding to auditory stimuli. Neurons in HVc, Area X, RA, and MAN of adult males are more responsive to the bird's own song than to other sounds, including conspecific and heterospecific song (Doupe, 1997). A common effect of opioids is an inhibition of neuronal activity (reviewed in Sarne *et al.*, 1996; Furukawa, Kotegawa, and Tsutsui, 1995). In humans, naloxone treatment increased

electrophysiological measures of selective auditory information processing (Arnsten, Neville, Hillyard, Janowsky, and Segall, 1984). Opioids may, therefore, inhibit non-selective neuronal responses to auditory stimuli in the VCRs, facilitating the discrimination of relevant acoustic information.

Finally, we cannot exclude the possibility that the amount of naloxone released by the minipumps was insufficient to induce behavioral and physiological effects. As was previously stated, no published study has documented effects resulting from the administration of naloxone via Alzet minipumps to a passerine bird. However, similar or smaller dosages in rats (0.95 mg/kg/hr: Jaloweic *et al.*, 1981) and mice (0.2 mg/kg/hr: Yoburn, Billings, and Duttaroy, 1993) decreased food intake and up-regulated opioid receptor densities, respectively. It is possible that dosages higher than those used in the present study are required to tonically antagonize opioid receptors in passerines. It is also conceivable that naloxone is metabolized and removed from the passerine circulatory system faster than in mammals, leading to decreased effectiveness. Naloxone binds with high affinity to both δ and μ receptor subtypes of the junco brain (Deviche, 1997), but we do not know what percentages of these receptors were occupied by the antagonist as a result of the experimental treatment.

Based on the information available to date, we conclude that the opioid system does not regulate song expression or VCR volume plasticity in adult songbirds. Further investigations are, however, necessary to elucidate the roles of this system in auditory processing, song learning, and neuroanatomical aspects of vocal control system development.

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Legend to Figures

Figure 1. Plasma Testosterone levels (means ± standard deviations) in adult male Dark-eyed Juncos exposed to either short days (SD) or long days (LD) and receiving naloxone-filled (NAL) or control (CON) mini pumps. Treatments were administered on Day 1. Stars indicate significant differences between photoperiods on a given day (Student Newman-Keuls, $p < 0.05$).

Figure 2. Volumes of HVc, Area X, RA, and MAN (means ± standard deviations) of naloxone-treated (NAL) or control (CON) male juncos exposed to either short (SD) or long days (LD). Differing letters indicate significant differences across groups (Student Newman-Keuls, $p < 0.05$).

Figure 3. Rt volume, telencephalon width, and brain weight (means ± standard deviations) of naloxone-treated (NAL) or control (CON) male juncos exposed to either short (SD) or long days (LD).

Figure 1.

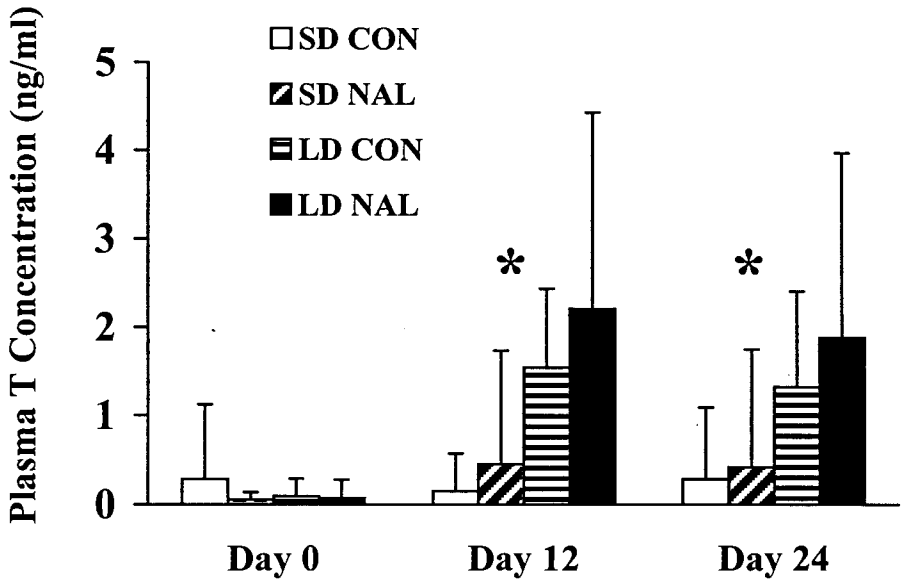
Plasma T Concentration (ng/ml)
5
4
3
2
1
0
SD CON
SD NAL
LD CON
LD NAL
*
*
Day 0
Day 12
Day 24

Figure 2.

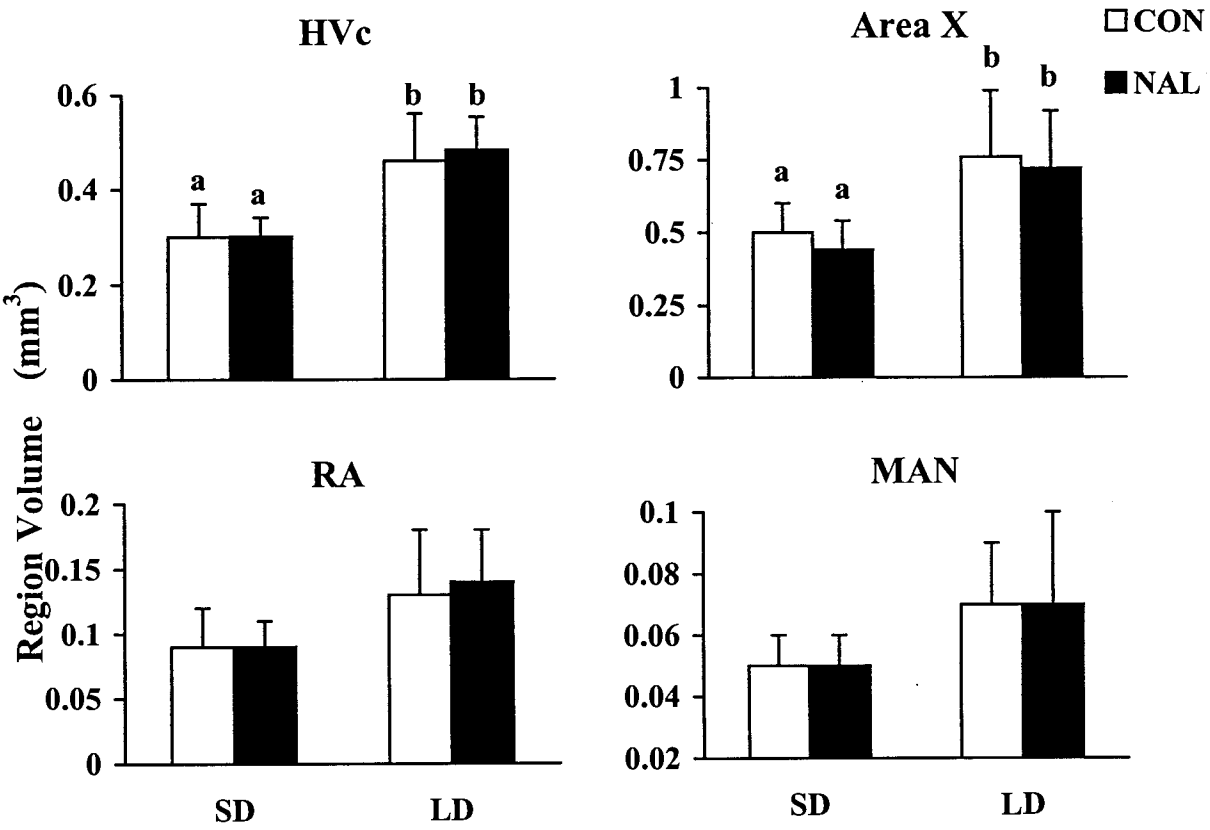
HVc
Area X
CON
NAL
RA
MAN
Region Volume (mm3)
0.6
0.4
0.2
0
1
0.75
0.5
0.25
0.2
0.15
0.1
0.05
0.08
0.06
0.04
0.02
a
a
b
b
SD
LD

Figure 3.

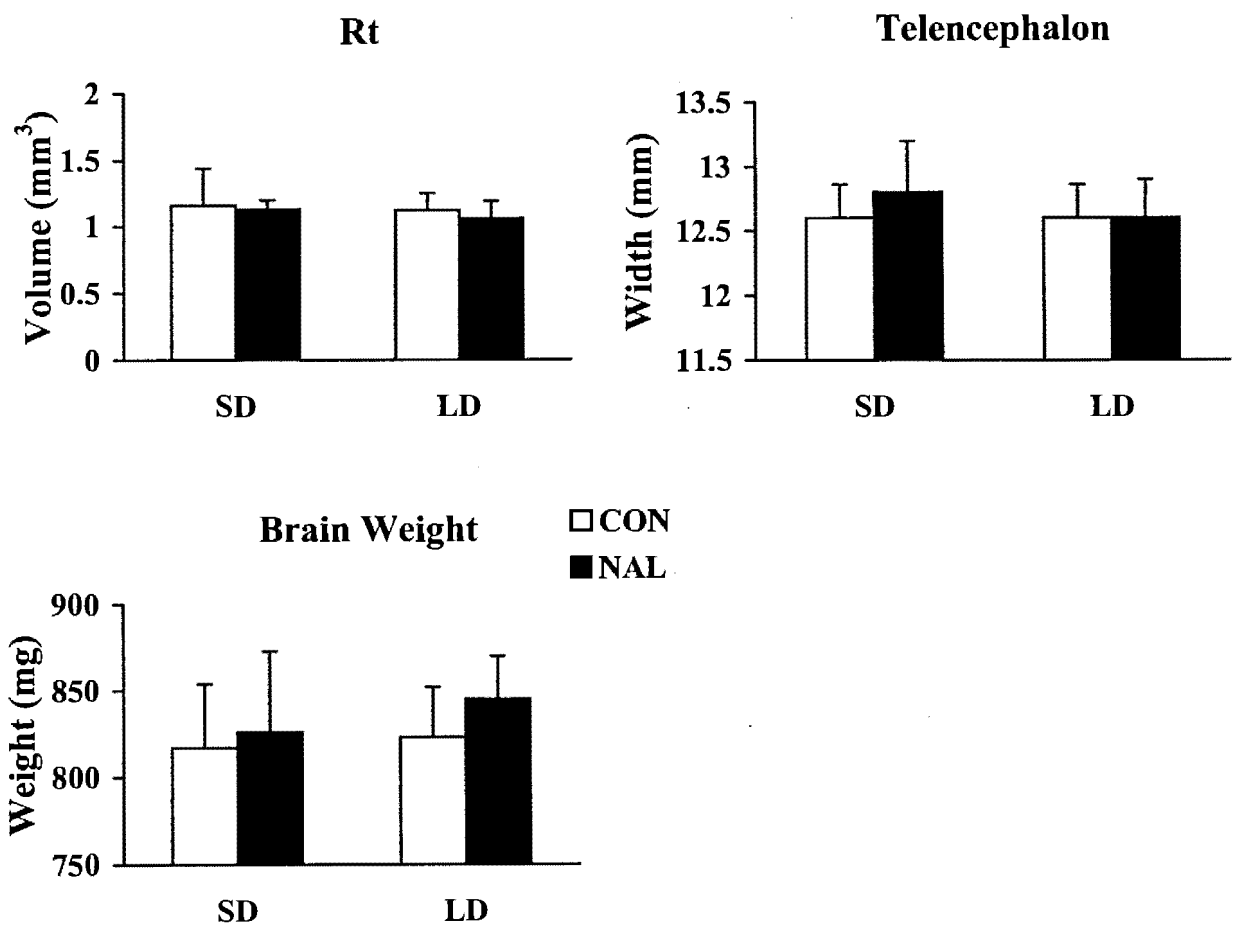
Rt
Volume (mm3)
2
1.5
1
0.5
0
SD
LD
Telencephalon
Width (mm)
13.5
13
12.5
12
11.5
SD
LD
Brain Weight
CON
NAL
Weight (mg)
900
850
800
750
SD
LD

Table 1. Body measurements over time for LD and SD birds receiving either naloxone or a control treatment for 25 days.

	Photoperiod	Treatment	Day 12	Day 24	ANOVA[1]
Fat Score	LD	C	1 ± 0.5	1 ± 0.5	n x t:
		NAL	1 ± 1	1.5 ± 1.5	p = 0.0441
	SD	C	2 ± 2	2 ± 1.5	
		NAL	1 ± 1	2.5 ± 1.5	
Body Mass (g)	LD	C	19.2 ± 0.7	19.2 ± 0.6[a]	p x t:
		NAL	18.6 ± 0.6	18.8 ± 0.4[a]	p = 0.0227
	SD	C	19.9 ± 1.7	20.7 ± 2.6[b]	
		NAL	20.1 ± 2.1	21.1 ± 3.2[b]	
CP Width (mm)	LD	C	5.87 ± 0.7[a]	6.63 ± 0.7[a]	photo:
		NAL	5.01 ± 1.0[a]	6.05 ± 1.3[a]	p = 0.0001
	SD	C	3.73 ± 0.4[b]	3.61 ± 0.4[b]	
		NAL	3.59 ± 0.3[b]	3.46 ± 0.4[b]	

Note: Fat score data expressed as medians ± 0.5 interquartile intervals.
Body mass and CP width expressed as means ± standard deviations.
[a,b]Different letters indicate significant differences on the specified date (SNK, $p < 0.05$).
[1]ANOVA p values: n x t indicates naloxone x time interaction, p x t indicates photoperiod x time interaction, and photo indicates photoperiod.

Table 2. Mean 24-Hour Food Consumption in grams.

Group	Days 8-10	Days 16-18	ANOVA
SD-Control	4.2 ± 0.8 a	3.7 ± 0.4 a	p = 0.0044
SD-Naloxone	4.3 ± 0.5 a	4.0 ± 0.3 a	
LD-Control	5.1 ± 0.7 b	5.8 ± 0.6 b	
LD-Naloxone	5.1 ± 0.5 b	4.7 ± 0.6 b	

Note: Data are expressed as means ± standard deviations. Different letters indicate significant differences within a column (SNK, $p < 0.05$). ANOVA p value refers to the effect of photoperiod.

Table 3. Frequencies of behaviors in long and short day-exposed juncos receiving either naloxone or control treatment.

	Short days		Long days		
Behavior	Control	Naloxone	Control	Naloxone	ANOVA
Singing	0^a	0^a	15 ± 22^b	36 ± 38^b	p = 0.0031
Feeding	20 ± 10^a	18 ± 7^a	15 ± 5^b	11 ± 2.5^b	p = 0.0280
Drinking	7 ± 2	6 ± 1.5	8 ± 3	6 ± 2	p = 0.1127
Preening	10 ± 6	5 ± 3	5 ± 3	7 ± 5	p = 0.1850
Locomotion	37 ± 14	40 ± 10	45 ± 10	38 ± 9	p = 0.4720

Note: All data are expressed as medians ± 0.5 interquartile intervals. Different letters indicate significant differences for a given parameter (SNK, $p < 0.05$). ANOVA p values refer to the effect of photoperiod.

Table 4. VCR Volumes and Total Brain Size of LD- and SD-exposed juncos after 25 days of naloxone or saline (control) treatment.

	Short days		Long days		ANOVA
	Control	Naloxone	Control	Naloxone	
HVc volume (mm^3)	0.30 ± 0.07a	0.30 ± 0.04a	0.46 ± 0.10b	0.48 ± 0.07b	p = 0.0001
Area X volume (mm^3)	0.50 ± 0.10a	0.44 ± 0.10a	0.76 ± 0.23b	0.72 ± 0.20b	p = 0.0001
MAN volume (mm^3)	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.07 ± 0.03	p = 0.0966
RA volume (mm^3)	0.09 ± 0.03	0.09 ± 0.02	0.13 ± 0.05	0.14 ± 0.04	p = 0.1133
Rt volume (mm^3)	1.16 ± 0.27	1.13 ± 0.07	1.12 ± 0.13	1.06 ± 0.13	p = 0.4982
Brain weight (mg)	817 ± 37	826 ± 47	823 ± 29	845 ± 25	p = 0.4633
Telencephalon width (mm)	12.6 ± 0.26	12.8 ± 0.40	12.6 ± 0.26	12.6 ± 0.30	p = 0.5920

Note: All data are expressed as means ± standard deviations. Different letters indicate significant differences within a given row (SNK, $p < 0.05$). ANOVA p values are for the effect of photoperiod.

IV. VOCAL CONTROL REGION VOLUMES, SONG PRODUCTION, AND PLASMA TESTOSTERONE DO NOT DIFFER WITH YEAR-CLASS IN CAPTIVE PHOTOSTIMULATED ADULT MALE DARK-EYED JUNCOS (*Junco hyemalis*)

ABSTRACT

Year-class comparisons in reproductive morphology, plasma testosterone (T), song production, and vocal control region (VCR) volumes were made in captive photostimulated adult male Dark-eyed Juncos (*Junco hyemalis*). We measured paired testis mass and cloacal protuberance (CP) width as indicators of reproductive condition, circulating plasma T, song rates, and the volumes of Area X, HVc, MAN, and RA in two year classes: SY (Second Year = males entering their first breeding season) and ASY (After Second Year = males entering at least their second breeding season). There were no differences in SY and ASY paired testes masses or plasma T levels, but SY males had smaller CP widths than ASY males as time after initial photostimulation increased. Although we found no difference in median song rates, we found our data to be inconclusive due to the fact that only 50% of the birds ever sang, and song rates among singers were highly variable. VCR volumes were similar in SY and ASY males, although SY males had heavier brains and greater telencephalon widths than ASY birds. Previous studies have shown year-class differences in plasma T, CP widths, and testis mass in free-living juncos. Our results indicate that VCR volumes and song production are independent of year-class, and they support the hypothesis that year-class differences in reproductive condition between SY and ASY juncos may be mediated by interactions with conspecific birds.

INTRODUCTION

In oscines, song learning and production are controlled by an interconnected set of brain nuclei (vocal control regions; VCRs) collectively called the song system, which exhibits neuronal plasticity throughout adulthood in all species studied to date (Nottebohm *et al.*, 1976, 1986; reviewed by Konishi, 1994; Smith, 1996; Brenowitz *et al.*, 1996; Gulledge and Deviche, 1997). Well-studied VCRs include the higher vocal center (HVc), Area X of the parolfactory lobe, the magnocellular nucleus of the anterior neostriatum (MAN), and the robust nucleus of the archistriatum (RA). HVc and RA are necessary for song expression (Nottebohm *et al.*, 1976), and Area X and MAN have been shown to be essential for song development (Nottebohm et al., 1976; Bottjer *et al.*, 1984; Sohrabji *et al.*, 1990; Scharff and Nottebohm, 1991).

In bird species breeding at high latitudes, such as Dark-eyed Juncos (*Junco hyemalis*), song production (as well as other reproductive activities) exhibits dramatic seasonal changes which coincide with seasonal changes in photoperiodic condition. Juncos sing during the breeding season, when they are photostimulated and plasma T levels are high. Singing stops when plasma T levels drop after the breeding season and the birds become photorefractory. In several species, singing is diminished or eliminated by castration, and subsequent T treatment reinstates the behavior (Arnold, 1975; Harding *et al.*, 1988). VCR volumes are also larger during than after the breeding season. Therefore, VCR volumes are large when birds are singing, plasma T is high, and the days are long. VCR volumes decrease when day length and plasma T decrease and birds stop singing (Smith, 1996; Brenowitz et al., 1996, Gulledge and Deviche, 1997). This seasonal pattern in VCR volumes has also been simulated in captivity (Nottebohm, 1981;

Brenowitz *et al.*, 1991; Smith *et al.*, 1995) by comparing adult male birds exposed to either SD or LD or given exogenous T treatment. The effects of T on VCR volumes are presumably mediated by the androgen receptors located in HVc, RA, and MAN (Arnold *et al.*, 1976; *Smith et al.*, 1996).

Previous studies have shown year-class differences in reproductive morphology and circulating levels of T in free living Dark-eyed Juncos (Deviche *et al.*, in press), as well as year-class differences in CP width, plasma T, and testes weight in free living Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*) (Morton *et al.*, 1990). In both cases, older adult birds had higher levels of plasma T, larger CP widths, and heavier testes than their younger counterparts. As of yet, no study has investigated whether these differences also occur in song production or VCR volumes. Because song production and VCR volumes are influenced by T, it is possible that year-class differences in plasma T levels will translate to differences in song production and VCR volumes as well.

The present investigation compares aspects of reproductive morphology and circulating plasma testosterone levels, as well as song production and VCR volumes in captive second-year (SY; birds hatched the previous year and entering their first breeding season) and older (After-second-year: ASY; birds having undergone at least one photoinduced gonadal cycle) photostimulated male Dark-eyed Juncos (*Junco hyemalis*). If VCR volumes and song production follow the pattern shown for reproductive morphology and plasma T in previous studies, we predict that SY birds will have smaller VCRs and sing less than ASY photostimulated males.

MATERIALS AND METHODS

Experimental design We collected 10 adolescent and 10 adult male Dark-eyed Juncos from a wild population near Fairbanks, Alaska (65° N, 148° W) in September, 1997. Birds were captured using seed-baited Potter traps. Birds were then housed in indoor group flight cages, given food and water *ad libitum*, and housed on a short day photoperiod (SD; 8L:16D) until March 11, 1998. At this time, birds were moved to individual cages which were visually, but not acoustically, isolated from one another. All birds were then gradually exposed to increasingly longer days, by adding one hour of light per day until 20 hours of light was reached (LD; 20L:4D). Birds remained on LD until they were killed on May 6.

Blood Samples and Testosterone Assay Blood samples were collected from the left alar wing vein 12 and 32 days after birds were transferred to LD. Samples were immediately centrifuged and plasma was drawn off and stored at -20° C until assay. Aliquots of plasma from each male at each sample time were assayed for total testosterone (T) using a coated tube ^{125}I kit obtained from Diagnostic Products Corp. (Los Angeles, CA). This assay has been used previously for measuring T in Dark-eyed Juncos (Gulledge and Deviche, 1998) and is both sensitive (lower detection limit: 10 pg/tube) and specific (cross-reactivity: 3% with dihydrotestosterone, 0.02% with estradiol). The intra- and inter-assay coefficients of variation for this assay are roughly 6% and 12%, respectively. All samples were assayed in duplicate in a single series and the averages were used for statistical analysis.

Morphological Measures In order to assess the reproductive condition of each bird throughout the experiments, we measured cloacal protuberance width (CP: a reliable indicator of reproductive system development (Schwabl and Farner, 1989; Deviche, 1992)) to the nearest 0.01 mm 12 and 32 days after initial exposure to LD. Also, at the time of sacrifice, testes were collected and weighed to the nearest mg.

Song Behavior The average song rate of each bird was quantified twice; between 7 and 10 and between 28 and 31 days after the onset of LD exposure. At both times, the same observer recorded the number of times each bird sang during two 30 minute periods. Time periods were randomly assigned to each bird, and all were between the hours of 6 and 11:30am (2 to 7 hours after lights on). The two counts of number of songs produced were then averaged at each time.

Brain processing and VCR volume measurement 40 days after exposure to LD began, all birds were killed by *in vivo* perfusion under complete anesthesia (methoxyflurane inhalation followed by transcardial injection of 0.2 M phosphate buffer and 4% buffered formalin). After perfusion, brains were collected, weighed to the nearest mg, and stored in a 30% sucrose solution for 4 days. Brains were then cryoprotected, frozen on powdered dry ice, and stored at -70° C. Brains were coronally sectioned (section thickness = 35μm) on a cryostat, and alternate sections were collected on gelatin-coated slides and stained for nissl substance using thionin. We used the MCID image analysis system (Imaging Research, St. Catherine, Canada) as previously described in Gulledge and Deviche (1998) to measure the volumes of four VCRs: HVc, RA, MAN, and Area X.

We also measured the volume of n. rotundus (Rt), a region not associated with the control of song, as a control nucleus. Regions were identified using the canary stereotaxic atlas (Stokes *et al.*, 1974; Nottebohm *et al.*, 1976). Lateral and medial MAN were measured together due to the difficulty distinguishing the boundary between them. Volumes of HVc were measured using the inclusive boundaries for the nucleus as described in Kirn *et al.* (1989). Telencephalon width was also measured to determine if overall brain sizes were different between groups. To do this, three sections with the anterior commissure present were chosen from each brain. The width of the telencephalon at the widest point on each section was then measured and averaged over the three sections.

All methods described were first approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks and also met the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical Analyses All data sets except song rates were normally distributed and homoscedastic. Song rates were therefore ranked prior to analysis. We compared testes weight and all brain measures between SY and ASY males using student's t-tests. CP widths, plasma T, and song rates were analyzed using one-way repeated measures ANOVA, followed by Student Newman Keuls multiple comparisons test if a significant time x age interaction was present.

RESULTS

Plasma T levels were not different between groups at either time, although the time x age interaction effect barely missed significance ($F_{1,14} = 3.8907$, $p = 0.0686$;

Figure 1.) It appears that, in captivity, SY birds have lower plasma T levels than ASY birds as the time since initial exposure to LD increases. CP width follows this same pattern, and the time x age interaction effect on CP width was significant ($F_{1,14} = 5.6859$, $p = 0.0318$; Figure 1.) CP width increased in both SY and ASY birds, but increased to a higher degree in ASY males. Mean CP width was significantly greater in ASY birds than SY birds on Day 33 (SNK, $p < 0.05$). Mean paired testes mass did not differ between groups (SY: 264 ± 106 mg; ASY: 261 ± 66 mg).

We found no effect of age or time on song rate. However, our results are not conclusive due to the fact that, during the course of the experiment, only 3 ASY and 5 SY birds ever sang, and these birds did not sing during every observation. During the first sampling period (Days 7 - 10), the median (± 0.05 interquartile interval) song rates for SY and ASY males were 5 ± 6 and 0 ± 0 songs / 30 min., respectively. During the second sampling period (Days 28 - 31), SY birds sang 6 ± 6 times in 30 min., and ASY birds sang 0 ± 5 times in 30 min.

All VCR volumes were similar in SY and ASY photostimulated male juncos (Table 1.). Volumes of Rt, a control nucleus, were also similar. SY males had heavier brains ($t_{1,14} = 16.54$, $p = 0.0012$), as well as greater telencephalon widths ($t_{1,14} = 13.51$, $p = 0.0025$) than ASY males (Table 1.). Due to this difference in brain size, we also analyzed VCR volumes that were adjusted for telencephalon width. These results were almost identical to those of the original data, therefore we only present the latter here.

DISCUSSION

The present investigation describes plasma T concentrations, reproductive morphology, song production, and VCR volumes in captive photostimulated adult male Dark-eyed Juncos of two year-classes: SY males experiencing their first cycle of gonadal development and regression and ASY males that have already undergone at least one such cycle. We found that CP widths were larger in ASY than SY males after 33 days of photostimulation. Plasma T levels, testes weights, and VCR volumes were similar in SY and ASY birds. We also found no difference in song rates, however, we do not feel that our data are conclusive. SY birds had heavier brains with greater telencephalon widths than ASY birds.

Previous studies have shown age differences in gonadal weights and plasma T concentrations in free-living Dark-eyed Juncos (Deviche *et al.*, in press; Ketterson and Nolan, 1992) and Mountain White-crowned Sparrows (Morton *et al.*, 1990). In all cases, older birds had higher plasma T levels and heavier gonads. Although not significant, our results indicate the same trend in plasma T levels in captive birds. We did see a difference in CP widths consistent with the results of Deviche *et al.* (in press). These minor discrepancies may be explained by the removal of social stimuli in our captive birds. Many other studies have shown effects of social interactions with conspecific young and other adults on reproductive physiology (Schwab and Lott, 1969; Dufty and Wingfield, 1986; Wingfield and Wada, 1989; Ketterson *et al.*, 1990; Wingfield *et al.*, 1994). Perhaps different year-classes are affected differently by social stimuli, hence removal of all social stimuli may affect them differently as well. These results do

indicate that careful consideration of data collected from captive versus free-living birds is necessary.

Due to the fact that SY and ASY birds in this study had statistically similar plasma T levels, we could not test the hypothesis that VCR volumes and song production varied with differences in plasma T. Plasma T levels of intact, captive, breeding male juncos never come close to reaching the levels of T measured in the blood of free-living breeding males (Deviche, unpublished observation). Also, as was previously stated, we do not feel that our song rate data are conclusive, and we will not discuss those results further. Therefore, we can not comment further on possible year-class differences in VCR volumes or song production. Due to the high variability in song rates and relatively low plasma T levels in captive birds, future studies of this kind should be conducted on free-living birds. By measuring song rates and plasma T levels of free-living birds, and then collecting them and measuring their VCR volumes, year-class questions could be better addressed, as well as kept in their correct environmental context.

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Legend to Figure

Figure 1. Plasma testosterone levels and cloacal protuberance (CP) widths in Second-Year (SY) and After-Second-Year (ASY) photostimulated male juncos. Data are expressed as means ± standard deviations.

Figure 1.

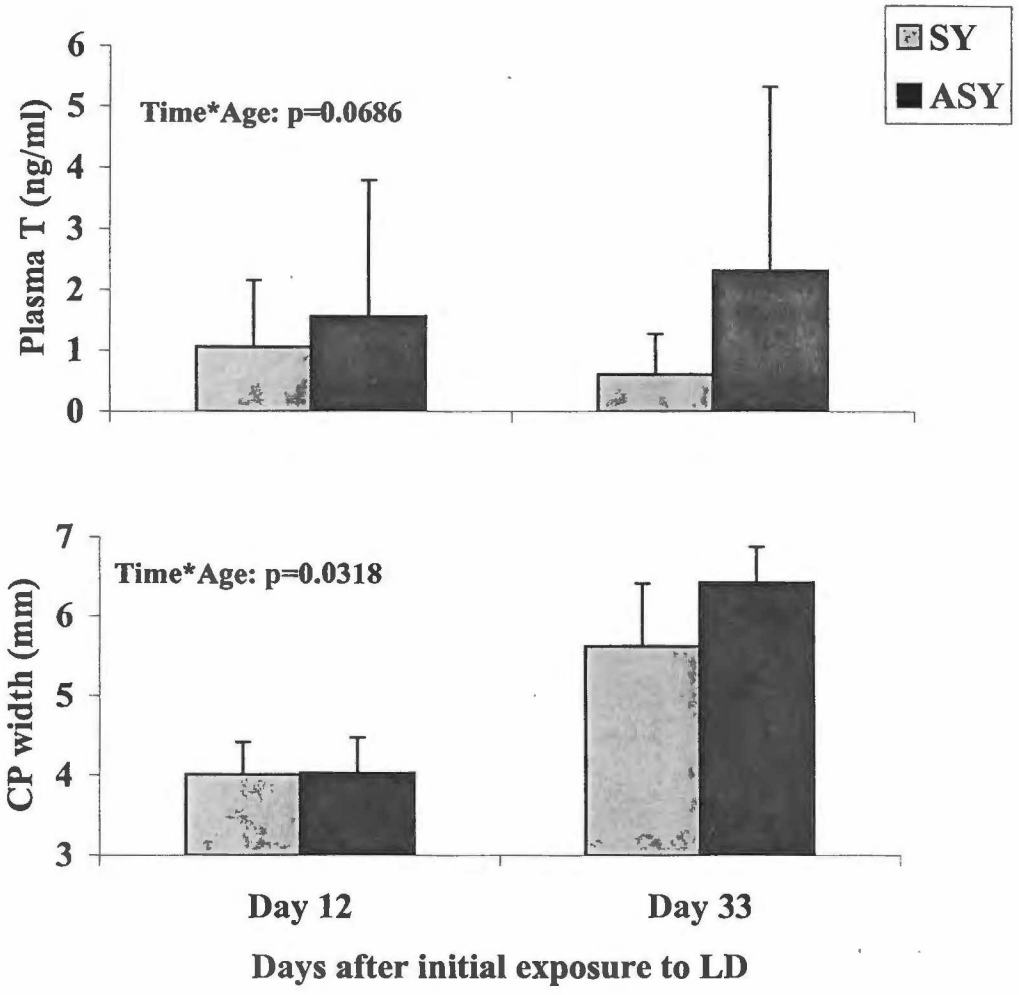
SY
ASY
Plasma T (ng/ml)
Time*Age: p=0.0686
CP width (mm)
Time*Age: p=0.0318
Day 12
Day 33
Days after initial exposure to LD

Table 1. A comparison of VCR volumes and brain morphology in Second-Year (SY) and After-Second Year (ASY) photostimulated male Dark-eyed Juncos. *

	SY	ASY	p value
VCR volumes (mm^3)			
Area X	0.73 ± 0.20	0.69 ± 0.17	0.6391
MAN	0.07 ± 0.02	0.05 ± 0.02	0.1050
HVc	0.45 ± 0.08	0.49 ± 0.12	0.4830
RA	0.16 ± 0.03	0.16 ± 0.03	0.7740
Rt volume (mm^3)	1.04 ± 0.14	1.04 ± 0.08	0.9535
Telencephalon width (mm)	12.67 ± 0.37	12.00 ± 0.37	0.0025
Brain weight (mg)	872 ± 39	795 ± 37	0.0012

* All data are presented as means ± standard deviations.

V. CONCLUSIONS

The major goals of this thesis were outlined in the introductory chapter. The following three chapters described experiments designed to meet these goals and the results provide new information on avian vocal control system function. Below I discuss what I believe to be the most important conclusions from my thesis work, as well as future directions to take within each of the areas studied.

Testosterone and Photoperiodic Condition

Chapter 1 dealt with the independent effects of testosterone (T) and photoperiodic condition, as well as effects of T which are modulated by photoperiodic condition, on song production and vocal control region (VCR) volumes. Previously, Nowicki and Ball (1989) showed that T-induced song production in Tree Sparrows (*Spizella arborea*) was modified by photoperiodic condition. This is not the case in Dark-eyed Juncos, because administration of T induces singing at similar rates regardless of photoperiodic condition. This could be due to a species difference or the different experimental designs of the two studies. We also found an independent effect of photoperiodic condition on the volume of HVc: photostimulation alone increased HVc volume and concurrent T administration did not increase it further. Previous studies by Smith *et al.* (1997) and Bernard *et al.* (1997) also indicated that exposure to LD increases HVc volume independent of T, but Smith *et al.* (1997) also showed an additional increase with T administration. Again, a different species (Gambel's White-crowned Sparrows, *Zonotrichia leucophrys gambelii*)

was used by Smith *et al.* (1997). Another major finding of chapter 1 is the fact that T is required for song production. Although our castrated, photostimulated male juncos had large VCR volumes, they never sang. Finally, chapter 1 shows for the first time that HVc still shows seasonal plasticity in castrated males.

Chapter 1 represents the first comprehensive study to date that simultaneously investigates song rates and VCR volumes in a wild passerine species across all photoperiodic conditions. The results are slightly different from previous studies on other species, indicating that species and experimental design differences should be taken into account before generalizing the results of a single experiment to all oscines. More comparative studies, as well as studies which investigate the mechanisms by which photoperiod itself affects VCR volumes, are needed.

Roles for Opioids

Opioid peptides (Ryan *et al.*, 1981; Ball *et al.*, 1988; Bottjer and Alexander, 1995; Deviche and Gunturkun, 1992; Carrillo and Doupe, 1995) and receptors (Gulledge and Deviche, 1995) are present within the VCRs. However, the function of the opioid system in the VCRs is unknown. Opioids do play a role in neuronal plasticity during development in birds (Meriney *et al.*, 1991), and opioid peptides reduce distress vocalizations in chicks (Panksepp *et al.*, 1987). In chapter 2, we investigated whether chronic opioid receptor blockade affected VCR plasticity or song production in adult male juncos. The results presented in chapter 2, together with the work of Gulledge and Deviche (1998), allow us to conclude that opioids are not involved in song production or

VCR volume plasticity. Additional studies are needed to determine the roles the opioid system has in avian vocal control. Specifically, future research on VCR opioids should focus on the effects of opioid receptor blockade on song learning and auditory processing.

Year-classes are Not Different

Finally, in chapter 3, we investigated whether there are year-class differences in VCR volumes or song production in captive adult male photostimulated juncos. We found no differences between second year and after second year males. Because we also did not see any differences in plasma T levels, which is the case in free-living birds, we suggest that any future studies on year-class differences in song or VCR volumes be done using free-living birds.

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