Published in final edited form as:

Gen Comp Endocrinol. 2008 August ; 158(1): 10–19. doi:10.1016/j.ygcen.2008.05.011.

Photoperiodic Induced Changes in Reproductive State of Border Canaries (*Serinus canaria*) are Associated with Marked Variation in Hypothalamic Gonadotropin-Releasing Hormone Immunoreactivity and the Volume of Song Control Regions

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Abstract

In temperate zone songbirds, such as canaries (*Serinus canaria*), seasonal variation in gonadal activity and behavior are associated with marked brain changes. These include gonadotropin-releasing hormone (GnRH) expression and the volume of brain areas controlling song production. Questions have been raised about the consistency of seasonal brain changes in canaries. Laboratory studies of the American singer strain raised doubts as to whether this strain exhibits a robust photoperiodic response along with changes in brain GnRH content, and studies of free-living canaries have failed to identify seasonal changes in volume of song control nuclei. We assessed differences in brain GnRH and the song control system associated with photoperiod-induced variation in reproductive state in Border canaries. We found that males and females maintained for 10 weeks on long days (14L:10D) regress their gonads, exhibit a decline in testosterone and initiate molt; a response consistent with the onset of absolute photorefractoriness (i.e., failed to respond to previously stimulating daylengths). All birds regained photosensitivity (i.e., exhibited gonadal response to stimulating daylengths) after experiencing short days (8L:16D) for 6 weeks. Furthermore, comparisons of birds in either a photosensitive, photostimulated, or photorefractory state revealed a marked increase in GnRH protein expression in the photosensitive and photostimulated birds over photorefractory birds. A similar variation was observed in the volume of key forebrain song nuclei. Thus, Border canaries demonstrate measurable neuroplasticity in response to photoperiodic manipulations. These data, along with previous work on other strains of canaries, indicate the presence of intra-specific variation in photoperiodically regulated neuroplasticity.

Keywords

Song bird; seasonal breeding; hormonal plasticity

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1. Introduction

Since the 1960s canaries have been one of the most popular songbird species in which to study seasonal changes in physiology, brain, and behavior and its photoperiodic control in captive-held birds (e.g., Steel and Hinde, 1966, Follett et al., 1973, Storey and Nicholls, 1978, Nottebohm, 1981). It is clear – based both on reports from field studies of wild populations and from many studies of a variety of strains of captive canaries – that this species breeds in a seasonal manner that is influenced by photoperiod. For example, canaries of the Border breed have small gonads when maintained on short days (e.g., 8L:16D) but they will exhibit a robust pattern of gonadal growth when transferred to long photoperiods (e.g., 16L:8D; Storey and Nicholls, 1978). Interestingly field studies reveal that canaries also exhibit flexibility in their timing of their reproduction in the sense that they can exhibit marked reproductive growth in response to non-photoperiodic cues while experiencing short days (Voigt and Leitner, 1998, Leitner et al., 2001). For example, within the Madeira archipelago, in years when rainfall is particularly heavy, wild populations of canaries will breed in December, a full six weeks prior to the usual onset of the breeding season and before photoperiod has increased to a duration above 12L:12D (Leitner et al., 2003).

Within the cardueline subfamily, that includes canaries, species variation in the degree of breeding flexibility is associated with species variation in the neuroendocrine plasticity in the hypothalamo-pituitary-gonadal axis (MacDougall-Shackleton et al., 2001, Hahn et al., 2004, Pereyra et al., 2005). Species in which photostimulation solely can instate vernal breeding activity tend to exhibit marked declines in preoptic-hypothalamus area during the non-breeding season of the key releasing peptide that controls gonadotropin function, gonadotrophin-releasing hormone-I (GnRH; e.g. Pereyra et al., 2005). Those species that can respond more opportunistically to a variety of cues tend to maintain appreciable GnRH throughout the year (e.g. Pereyra et al., 2005). Despite many years of study, there is disagreement as to whether seasonal changes in reproductive activity are associated with such plasticity in the neuroendocrine system of canaries, perhaps because of the failure to recognize the flexibility of canary breeding strategies. For example, a report on the American Singer canary breed (Bentley et al., 2003) suggested that when this strain is chronically photostimulated they do not exhibit absolute photorefractoriness -- defined by a regression of the gonads after prolonged photostimulation that cannot be reversed even with 24 hours of light (Hamner, 1968). Additionally, GnRH content in the brain does not change in these birds as they transition from reproductive to non-reproductive conditions (Bentley et al., 2003), as it does in many absolutely refractory species both within and outside the cardueline subfamily (e.g., Dawson et al., 1985, Goldsmith et al., 1989, reviewed in Ball and Hahn, 1997). As discussed in Bentley et al. (2003), it is possible that this breed has lost a wild-type pattern of photoperiodic responsiveness along with the associated neuroendocrine plasticity, but this cannot be asserted in the absence of knowledge about plasticity in GnRH in other canary populations. A second aspect of seasonal neuroendocrine plasticity relates to seasonal changes in the volume of song control nuclei. This phenomenon was discovered in the Wasserschlager breed of canaries by Nottebohm (1981), who noted that the volume of several regions is far larger in birds collected in the spring than in birds sampled in the fall. This observation has subsequently been replicated in other domesticated canary breeds (e.g., Nottebohm et al., 1986, Bottjer and Dignan, 1988, Brown and Bottjer, 1993, Fusani et al., 2000), and extended to a substantial number of other temperate zone songbird species (see Ball, 1999, Tramontin and Brenowitz, 2000 for reviews). However, one of the few field studies of wild canaries sampled birds at different times of year and failed to find differences in song nuclei volume (Leitner et al., 2001).

Studies in the 1970s were based on the assessment of gonadal size and peripheral endocrine measures indicated that Border canaries exhibit a robust response to photoperiod including the development of absolute photorefractoriness similar to that described in other seasonally

breeding songbird species (Follett et al., 1973, Storey and Nicholls, 1976;1978, Nicholls et al., 1988). However, no studies of brain changes associated with differences in seasonal state have been investigated in this breed. Brains from Border canaries were therefore collected when they were in one of three reproductive states: photosensitive, photostimulated or photorefractory. We measured GnRH immunoreactivity and the volume of key forebrain song control nuclei such as HVC, the robust nucleus of the arcopallium (RA) and area X of the medial striatum in both males and females. We find clear evidence of marked neuroplasticity associated with photoperiodically induced variation in reproductive state both in the GnRH system and in the song control system in Border canaries.

2. Experimental Procedure

2.1. Animals

Border canaries maintained on natural day length were obtained from a local supplier (Maryland Exotic Birds) in June 2004, sexed by laparotomy, and segregated into single sex cage groups of not more than 5 birds (N=40, 18 males, 22 females). However, the sexes were not visually or acoustically isolated from one another. They were then housed on short days (8L:16D) for 6 weeks to insure that they were all in a photosensitive reproductive state before the start of the study.

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and with the approval of the Johns Hopkins University Animal Care and Use Committee.

2.2. Procedures used to induce variation in reproductive state

In this study, the following procedures were used to define reproductive state. These are based on generally agreed definitions of reproductive state in canaries and other birds (e.g., Storey and Nicholls 1976; Nicholls et al. 1988; Dawson et al., 2001). Birds were deemed to be photosensitive when maintained on short daylengths (6L:18D) for a minimum of 6 weeks and gonads were small. Photostimulation was established when photosensitive birds were shifted to long daylengths (14L:10D) and the gonad recrudescenced. The birds were considered photorefractory when gonadal size had started to regress and molt was initiated – two events indicative of the onset of photorefractoriness (molt was scored using criteria recommended by Dawson and Newton, 2004). Additional measures of endocrine physiology, neuroendocrine system, and the song control system were taken as described within the separate experiments.

2.3 Experiment One

Animals—Following 6 weeks of exposure to short days (8L:16D) all birds were moved to long days (14L:10D) for 18–19 weeks (variation caused by separation of individuals into discrete phototreatment groups for experiment 2). Supplementary lights were added after 6 weeks because it is known that overhead lighting can result in unequal lighting intensity when using multi-stack cages (Bentley et al., 1998). Cage stacks used had 3 levels: no significant difference in early gonad development was found between levels ($p > 0.256$). Food and water were provided *ad libum*. Nest cups and nesting material were not provided.

Reproductive state determination—Exploratory laparotomies were conducted at weeks 4, 7, 11, and 17 on males and weeks 4, 8, 12 and 17 on females to assess the bird's breeding condition by measuring the size of their gonads. Birds were anesthetized by injecting .05–.08ml of 15mg/ml secobarbital into their breast muscle. A small incision was then made below the last rib on the left side to measure diameter of the left testis or largest follicle. In males the diameter of the testis was measured with calipers directly or by comparison of a marked angle tipped probe to gonad. For females, follicle volumes were calculated using the formula for a

sphere, $V=4/3\pi r^3$, where V = volume and r = follicle radius. The volume of the left male testis was determined at the end of the study after brain collection and was calculated using the formula for a prolate spheroid, $V=4/3\pi a^2b$, where 'a' is half the width (short axis) and 'b' is half the length of the testis. The size of follicles in females determined to be in lay were estimated directly from the yolk of freshly laid egg, due to increased risk of rupturing a follicle internally during laparotomy. Blood samples were collected from the alar (wing) vein one week prior to laparotomies for hormone analysis. The number and size of eggs laid by individual females was noted, as was the size extent of brood patch edema (based on Hinde, 1962), a sign of an actively nesting female. Molt of both sexes was scored to determine progression towards photorefractoriness (using Dawson and Newton, 2004 schema).

2.4. Experiment Two

Animals—After it was determined that the birds in experiment one had become refractory, they were randomly divided into three treatment groups: photosensitive, photostimulated, and photorefractory. One third (6 females and 6 males) of the birds were left on long days (photorefractory group) while the remaining birds (11 males and 11 females) were returned to short daylengths (8L:16D) for 6 weeks (photosensitive and photostimulated groups). After 6 weeks the photostimulated group (6 females and 6 males) was placed back on long days (14L:10D) for 4 weeks before all groups were sacrificed.

Perfusions—Prior to perfusion, birds were injected with 0.05ml heparin (20 mg/ml im), and then euthanized with the administration of an overdose of secobarbital (.06–.10 ml). The birds were assessed as near death when they no longer responded to painful stimuli (i.e. toe pinch), at which time they were weighed and prepared for pericardial perfusion. The perfusion was completed by circulating 0.9% saline (Na-PBS) until the external flow ran clear, then 4% paraformaldehyde (in 0.1M PBS pH 7.4) was run through for approximately 5 min to fix the tissue. The brain was then extracted and post-fixed in paraformaldehyde for at least 1 hr. Brains were then cryoprotected for 2 nights in a sucrose solution (30% wt/vol sucrose in 4% paraformaldehyde). After post-fixation the brains were frozen on dry ice and stored at -70°C until processed.

Brain Processing—Brains were sliced in the coronal plane on a cryostat (Micron HM 500 OM) at $40\mu\text{m}$ and sections collected alternately into four cell culture trays with cryoprotectant (per liter: 500ml 0.1M PBS pH 7.2, 300g sucrose, 10g polyvinylpyrrolidone, and 300ml ethylene glycol) to create four separate series for differential analysis. Thus, when the sections from an individual series were analyzed for any given assay they were $120\mu\text{m}$ apart. Trays were then stored at -20°C until processed for histological analyses. Sections were either Nissl-stained (thionin, Sigma Chemical, St.Louis, MO) or processed for the localization of GnRH with an immunocytochemical procedure. Brains were randomly ordered for slicing so that one male and one female from each treatment were sliced and processed as a group ($n=6$) to control for assay variance.

Immunocytochemistry—One series of sections from each canary brain was processed using rabbit-raised antibody to GnRH (HU60 bleed F - generous gift of Dr. Henryk Urbanski, OHSU) in a single label immunocytochemistry (ICC) reaction. This antibody reacts only to the intact decapeptide of GnRH-I and GnRH-II in mammals, salmon, and birds (Urbanski, et al., 1990, Urbanski, 1992). It has been used successfully for over 10 years to localize GnRH in a variety of songbird species, including canaries (e.g. Hahn and Ball, 1995; Bentley et al., 2003). Free-floating sections were processed as described in Bentley et al. 2003, with the exception that the GnRH primary was used at 1:2500 (concentration determined by dilution series to provide best visualization with lowest background) and only incubated overnight.

Section Analysis—Sections were visualized using a light microscope (Zeiss Axioskop, Carl Zeiss, Thornwood, NY) mounted with a video camera (Zeiss Axiocam, Carl Zeiss, Thornwood, NY). The camera image was digitized using Open Lab system firmware/software (Improvision Openlab version 3.5, Lexington, MA).

Volume reconstruction of key telencephalic song nuclei (HVC, RA and Area X) and two reference nuclei (Rt and PT) was calculated based on an analysis Nissl-stained tissue using Openlab software. Boundaries of the nuclei were traced, and volume was computed by summing the defined areas, accounting for distance between sections. Volume measurements from just one side were used for statistical analysis. We statistically tested for left-right asymmetries and found no significant differences between the left and right song nuclei.

Variation in the number of cells expressing immunoreactivity for the GnRH protein was compared by counting the number of cell bodies in the pre-optic area (POA), a brain region known to exhibit seasonal variation in the expression of this peptide. This is also the anatomical localization of the GnRH-I populations of cells that are known to be the primary regulator of gonadotropin release in birds. Cells in every brain section – which were 120µm apart due to alternating series collections – containing the POA were counted. The POA was defined as starting just posterior to the prominent split in the Septopallio-mesencephalic tract (TSM) that is associated with rostral part of this area and ending at the anterior commissure. This cell counting was conducted at 200x magnification in a single plane focus. The identities of cells were confirmed at 400x if needed (magnification = 20x or 40x lens plus a 10x viewer). Overall immunoreactivity was assessed through a custom macro within ImageJ, which calculated relative optical density per unit area after the removal of background staining in a 500×500 pixel box oriented to the right of midline (in the POA) on sections containing POA. This resulted in an average percentage expression, which was converted using an arcsin equation so that the fundamental distribution was virtually normal and the statistical assumption of normal distribution was not violated (Zar, 1974).

At all times the individual birds were referenced only by their number and processed blind to their sex and treatment group to ensure unbiased collection of data.

Hormone Analysis—A radioimmunoassay (RIA) was conducted on plasma fractions maintained from date of draw at –20°C, to determine the level of testosterone in male and female birds using a MP Biomedicals Double Antibody Testosterone ¹²⁵I kit (Costa Mesa, CA, USA). This assay was highly sensitive (i.e., 25 pg/ml) and specific (i.e., cross-reactivity to 5α-Dihydrotestosterone (DHT) is 3.4%, and to 17β-estradiol and corticosterone < 0.01%). Directions of the kit were followed as stated, except the titration curve was diluted to .0025, 0.05, 0.1 and 0.25 ng/ml beyond the normal 0.5, 1.0, 2.5, 5.0, 10.0 ng/ml standards to account for the low levels of testosterone in avian blood using protocols previously established in our lab (Duffy, et al., 2000, Young, et al., 2001, Sartor and Ball, 2005). All samples were run in a single assay.

Statistics—A two-way Analysis of Variance (ANOVA) was employed to test the effects of the two factors, photoperiodic state and sex, as well as their interaction. This test was employed for measures taken in both sexes, i.e. GnRH-ir, GnRH cell size, as well as the volumes of the three song control nuclei HVC, RA and area X. For variables for which measures were specific to a single sex (i.e., gonadal size and testosterone concentrations) a one-way ANOVA was applied for the data analysis so that the measures for males or females collected in different reproductive states could be compared. These analyses were followed by post-hoc tests such as the Tukey's HSD test.

3. Results

3.1. Experiment One

Gonad Development—Both males (figure 1a) and females (figure 1b–c) exhibited the expected gonadal response to photostimulation. In males, a repeated measures ANOVA revealed a significant difference in testis size over the course of the photoperiodic treatment ($F_{4,65}=38.121$, $p<.0001$). Significant increases in size could be detected by week 4 ($p<.01$). As they were maintained on long days and became photorefractory, testis size returned to sizes not significantly different from pre-photostimulation values. Similarly, ovarian volume changed significantly in females as they experienced long days and made the transition from being photostimulated to becoming photorefractory. As expected, not all of the females exhibited increase in follicle development as extreme as is apparent in the males' testis development. Females songbirds, even ones that live in captivity such as canaries, generally require secondary cues to stimulate egg laying. Therefore, females tend to exhibit more variance than males in gonadal growth in response to photostimulation alone. All females exhibited brood patch development, but only those that built nests in food cups and laid eggs fully developed vascularisation and edema. Therefore, the data from females are presented separately as layers ($N=7$, figure 1b) and non-layers ($N=10$, figure 1c). In both cases there were significant changes in ovarian volume over the course of the study (layers: $F_{4,35} = 11.53$, $p<.0001$; non-layers $F_{4,45} = 2.715$ $P<.04$).

Molt—Molt started prior to week 11 of photostimulation, and was nearly complete by week 16. At this time all birds had fully regressed their gonads.

Testosterone Levels—Males exhibited a marked increase, followed by a sudden decrease, in plasma testosterone concentrations that is similar to the pattern of testis growth and regression (figure 2). There was a significant difference in plasma testosterone concentrations in the expected direction among the males in the three different treatment groups as assessed at the time of brain collection at the end of the experiment ($F_{(2,13)}=4.28$, $p=0.045$). Photostimulated males exhibited concentrations nearly an order of magnitude higher than the photostimulated and photorefractory groups.

The females exhibited relatively low levels of testosterone once photostimulated, but showed a brief increase after becoming refractory. The peak level of female testosterone is in the range of testosterone concentrations measured in photosensitive males (Data not shown).

2.3. Experiment Two

Measures of GnRH protein immunoreactivity—The results of the quantitative analysis of GnRH immunoreactivity are presented in figure 3. Representative photomicrographs of the pattern of staining in males in three different reproductive states are presented in figure 4. Both the percentage of area exhibiting GnRH immunoreactivity and the number of GnRH immunoreactive cells were compared with the use of a two-way ANOVA with sex and reproductive state as the two factors. No significant effect of sex was observed in either the case of the percentage of area exhibiting immunoreactivity ($F_{1,25} = 0.034$ $p=0.855$) or the number of immunoreactive cells ($F_{1,25} = 0.186$ $p=0.670$; Figure 3, Figure 4). In the case of both measures though there was a significant effect of reproductive state alone (percentage area: $F_{2,25} = 5.901$, $p=.008$; cell number $F_{2,25} = 11.62$ $p <.001$). Photostimulated birds exhibited the highest values for both measures and in both cases they are significantly larger than the photorefractory birds based on post-hoc testing ($p= 0.006$ percentage area; $p<0.001$ cell number). In the case of cell number, there was also a significant difference between the photosensitive and the photostimulated groups ($p = 0.003$) but this was not the case for the

measure of percentage area of immunoreactivity ($p = 0.063$). No significant interaction between the variables of reproductive state and sex was observed based on an analysis of both measures.

Song Nuclei Volume—Bar graphs presenting the mean volume for the three song control nuclei measures are presented for males and female in the three different reproductive states in figure 5. Photomicrographs illustrating the three nuclei in Nissl stained material are presented in figure 6. Based on the results of a two-way ANOVA for each nucleus an expected significant sex difference in volume was identified for each brain area (HVC: $F_{1,24} = 94.94$, $p < 0.001$; RA: $F_{2,24} = 64.3$, $p < 0.001$; area X: $F_{1,24} = 93.938$, $p < 0.001$). There was as a significant effect of reproductive state both in the case of HVC ($F_{2,24} = 4.352$, $p = 0.024$) and RA ($F_{2,25} = 22.6$, $p < 0.001$). There was not a significant difference among birds collected in the three different reproductive states in the case of area X ($F_{2,24} = 1.99$, $p = 0.158$). For both HVC and RA there was a significant sex by group (reproductive state) interaction (HVC: $F_{2,24} = 6.05$, $p = 0.007$; RA: $F_{2,25} = 29.08$, $p < 0.001$) and not significant interaction was observed for area X ($F_{2,24} = 2.76$, $p = 0.083$). Post-hoc testing revealed that in HVC, the photosensitive and photostimulated birds were not different from each other but the photostimulated birds were significantly larger than the photorefractory birds ($P = 0.034$). For RA the stimulated birds were larger in volume than the photorefractory and the photosensitive birds ($P < 0.0001$).

Reference Nuclei Volume: There was no significant difference between or within sexes, treatments, or interactions for either reference nuclei: Nucleus rotundus (all p 's > 0.06) and Nucleus pretectalis (all p 's > 0.420)

4. Discussion

The photoperiodic manipulations we performed on male and female Border canaries in this study produced the expected effects on measures of reproductive activity. When placed on long daylengths photosensitive males and females exhibited a dramatic growth in gonadal size, however, when maintained on these long daylengths for six weeks or more they exhibited a spontaneous regression of their gonads (i.e., gonadal regression while the photoperiod stayed constant) along with the onset of molt. This pattern of response to daylength has been previously described for this strain of canary (Follett et al., 1973, Storey and Nicholls, 1976; 1978, Nicholls et al., 1988) and is indicative of a species exhibiting a pattern photorefractoriness known as absolute photorefractoriness (Farner et al., 1983, Nicholls et al., 1988, Dawson et al., 2001).

In addition to discerning a response to photoperiodic manipulations that is consistent with canaries exhibiting a pattern of absolute photorefractoriness, we also observed marked neuroplasticity in two neuroendocrine systems: 1) the preoptic-hypothalamo GnRH systems that regulates the release of gonadotropin hormones from the pituitary and 2) the forebrain song control circuit that regulates seasonal changes in song behavior. Specifically we found that photosensitive and photostimulated canaries tended to exhibit higher amounts of GnRH immunoreactivity than birds in a photorefractory state (figure 3, figure 4). Male canaries collected in these different reproductive states also exhibited marked differences in the volume of key song control nuclei such as HVC and RA (figure 5 and figure 6). Males in the photostimulated conditions had volumes of HVC and RA significantly larger than photorefractory males. Interestingly, photosensitive males had an HVC volume not different from the photostimulated birds. Females in this species are known to exhibit volumes of song nuclei substantially smaller in size than those of males (Nottebohm and Arnold, 1976) and we observed this difference in this study. In the females there was no variation in volume associated with differences in reproductive state in contrast to what was observed in males (Figure 5). Previous studies in the laboratory (e.g., Bentley et al., 2003) and in the field (Leitner et al., 2001) have raised questions about whether the song system and the GnRH system do indeed

exhibit adult seasonal plasticity in canaries. The reason we observed these differences should be discussed in the context of these previous studies of canaries as well of other songbird species.

4.1. Seasonal Changes in the GnRH in Relation to Variation in Breeding in Canaries

We investigated variation in cells immunoreactive for GnRH-I. This form of the decapeptide has been clearly linked to the control of gonadotropin secretion in birds and has been found to exhibit a marked variation in several other species collected in different physiological states (e.g., Dawson et al., 1985, Ball and Hahn, 1997 for a review). Not all species exhibit such variation in GnRH content in relation to reproductive state (see Ball and Hahn, 1997, Dawson et al., 2001, Pereyra et al., 2005). Initially it appeared as if the degree of seasonal GnRH plasticity was associated with variation in the onset of photorefractoriness (Ball and Hahn, 1997, Hahn et al., 1997), but recent work has defined a set of general rules influencing plasticity (Pereyra et al. 2005). In particular, comparative studies of cardueline finches with diverse photoperiod response patterns suggest that GnRH plasticity tends to be observed in species that do exhibit gonadal regression while still experiencing long daylengths that were previously able to stimulate gonadal growth (Pereyra et al. 2005, Macdougall-Shackleton et al., 2006). As demonstrated here, Border canaries fit this pattern. Males and female maintained on long days regress their gonads and when sampled in different reproductive states they exhibit marked variation in the hypothalamic content of GnRH. How general are these findings? A previous study in the American singer breed of canaries found that they failed to regress their gonads while maintained on long days and that they also failed to exhibit plasticity in the GnRH system (Bentley et al., 2003). Thus, intra-specific variation in responsive to photoperiod in relation to GnRH plasticity among canary breeds is similar to inter-specific variation within the cardueline sub-family. An important question is whether this plasticity occurs in wild populations of canaries. There have been several field studies of seasonal cycles of reproduction in wild canaries on islands in the Madeiran archipelago (e.g., Voigt and Leitner, 1998, Leitner et al., 2003) but variation in the GnRH system was not investigated. Overall the pattern of breeding that has been observed fits in well with other cardueline finches that are clearly photoperiodic, but exhibit flexibility in the timing of breeding depending on the availability of supplementary cues. By flexibility in this case we mean breeding at times of year when daylengths are still short (e.g., early winter) and one might not assume an obligatory photoperiodic species would breed. However, many apparently flexibly breeding species still exhibit clear variation in reproductive state, i.e. photosensitive, photostimulated or photorefractory that is governed by variation in photoperiod (Hahn et al., 1997). In these species, the reproductive axis is “switched on” when they are on short days and photosensitive, thus they are able to respond to many different cues in addition to long day lengths. This appears to be the case in canaries. Based on the data collected here, experiencing short days dissipates photorefractoriness and leads to an up-regulation of GnRH. In wild canary populations, birds that are photosensitive but experiencing short days can respond to increases in rainfall, for example in December and start growing their gonads well before long day lengths occur (Leitner et al., 2003). Laboratory studies have demonstrated that this effect of rainfall is mediated by an increase in green plants as captive birds on short days characteristic of December will breed earlier when experiencing green plant material than when experiencing rainfall alone (Voigt et al, 2007). These observations of flexible reproduction in wild canaries that are photosensitive and on short days are very consistent with observations of other species in the cardueline group (e.g. Macdougall-Shackleton et al., 2006). What would be interesting to know is if any cue could elicit breeding in wild canaries at the end of the summer when they are presumably photorefractory and have a decrease in the expression of GnRH in the preoptic area-hypothalamus.

4.2. Seasonal Changes in the Song Control System of Canaries and other Songbird Species

A second example of seasonal neuroendocrine plasticity observed in this study concerns variation in the volume of forebrain song control nuclei associated with variation in reproductive state. Substantial changes in the volume of nuclei such as HVC, area X and RA was first described by Nottebohm (Nottebohm, 1981) when he collected captive canaries held on natural photoperiods either in April when they were photostimulated and in full reproductive condition, or in September when they were almost assuredly still photorefractory. Thus, his groups are comparable to the photostimulated and photorefractory groups in our study and our results of significant differences in the volume of two forebrain song nuclei, HVC and RA, are consistent with his previous observations. Several other laboratory studies of canaries involving either the manipulation of photoperiod or of circulating testosterone concentrations have also found that long photoperiods and high testosterone tend to be associated with large volumes of HVC, RA and area X while low to undetectable concentrations are associated with significantly smaller volumes of these song nuclei (Nottebohm, 1980, Nottebohm et al., 1986, Brown and Bottjer, 1993, Johnson and Bottjer, 1993, Sartor et al., 2005). Recently, two field studies of song sparrows (Tramontin, et al., 2001) and blue tits (Caro et al., 2005) respectively have found that the volume of song nuclei can reach full adult breeding size well before the reproductive system as completely recrudesced and plasma testosterone concentrations are maximal. These studies suggest that the song system is sensitive to quite low concentrations of testosterone. The data collected in our study of Border canaries supports this idea given that males in the photosensitive state with measurable but relatively low testosterone concentrations had HVC volumes comparable to those of males who were photostimulated and in full reproductive condition. However, it should also be noted that other factors could contribute to increases in the volume of song nuclei in canaries independently of testosterone such as exposure to a female (Boseret et al., 2006). Also, the act of singing promotes the release of the neurotrophin brain-derived neurotrophic factor (BDNF; Alvarez-Buylla and Garcia-Verdugo, 2006), which increases the probability that new neurons will be incorporated into HVC (Li et al., 2000, Alvarez-Buylla and Garcia-Verdugo, 2006). The males in this study were exposed to females and their rate of singing was not controlled so we can not dismiss the possibility that these factors contributed to the large volumes of the song nuclei we measured in the short day photosensitive with relatively low testosterone concentrations.

Our studies also support the notion that photoperiodic manipulations that place birds in different reproductive states are associated with differences in the volume of song nuclei. Just as with our consideration of GnRH plasticity, a question arises concerning whether the plasticity we observed in the song system occurs in the natural situation. A field study of male canaries captured on the Madeira islands showed that HVC and RA volumes in males collected in late April/May in breeding condition (with relatively high plasma testosterone concentrations) were not different from non-breeding males collected in either October (with undetectable testosterone concentrations) or December (with low but detectable testosterone concentrations). The birds collected in October may well of have still been in the photorefractory state, while those collected in December should be photosensitive, thus this study is not strictly comparable to ours. The non-breeding birds would have to be subdivided into animals that are clearly photorefractory or clearly photosensitive for us to compare these results and assess whether there is a discrepancy. If we had lumped the photorefractory and short day photosensitive birds into one category we also would probably have not detected a significant difference between the two groups. It is also the case that in the Leitner et al. (2001) study factors such as social stimuli or song rate may have maintained high volumes of the song nuclei in the non-breeding seasons even though testosterone concentrations are low to undetectable in these groups.

4.3. Conclusions the value of intra-specific variation for genetic studies

Our work when placed in the context of studies on other breeds of canaries demonstrates how seasonal plasticity in both the hypothalamo-pituitary-gonadal axis and in a forebrain system that controls vocalizations can be lost through the process of domestication. Given that this strain variation in canaries reflects species variation in the carduelines this phenomenon provides a good opportunity to study genetic changes that underlie species variation in breeding flexibility and the associated neuroplasticity.

Acknowledgments

The authors would like to thank Henryk Urbanski for his generous donation of the HU-60 anti-body. Funded by: NIH/NINDS (RO1 NS F35467) to GFB.

References

- Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. *J Neurosci* 2002;22(3):629–634. [PubMed: 11826091]
- Ball, GF. Neuroendocrine basis of seasonal changes in vocal behavior among songbirds. In: Hauser, M.; Konishi, M., editors. *The Design of Animal Communication*. MIT Press: Cambridge; 1999. p. 213–253.
- Ball, GF.; Hahn, TP. GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In: Parhar, IS.; Sakuma, Y., editors. *GnRH neurons: Gene to behavior*. Tokyo: Brain Shuppan Publishers; 1997. p. 325–342.
- Bentley GE, Goldsmith AR, Dawson A, Briggs C, Pemberton M. Decreased light intensity alters the perception of day length by male European starlings (*Sturnus vulgaris*). *J Biol Rhythms* 1998;13(2):148–158. [PubMed: 9554576]
- Bentley GE, Audage NC, Hanspal EK, Ball GF, Hahn TP. Photoperiodic response of the hypothalamo-pituitary-gonad axis in male and female canaries, *Serinus canaria*. *J Exp Zool A Comp Exp Biol* 2003;296(2):143–151.
- Boseret G, Carere C, Ball GF, Balthazart J. Social context affects testosterone-induced singing and the volume of song control nuclei in male canaries (*Serinus canaria*). *J Neurobiol* 2006;66(10):1044–1060. [PubMed: 16838373]
- Bottjer SW, Dignan TP. Joint hormonal and sensory stimulation modulate neuronal number in adult canary brains. *J Neurobiol* 1988;19(7):624–635. [PubMed: 3225559]
- Brown SD, Bottjer SW. Testosterone-induced changes in adult canary brain are reversible. *J Neurobiol* 1993;24(5):627–640. [PubMed: 7686962]
- Caro SP, Balthazart J, Thomas DW, Lacroix A, Chastel O, Lambrechts MM. Endocrine correlates of the breeding asynchrony between two Corsican populations of blue tits (*Parus caeruleus*). *Gen Comp Endocrinol* 2005;140(1):52–60. [PubMed: 15596071]
- Dawson A, Newton I. Use and validation of molt score index corrected for primary-feather mass. *Auk* 2004;121(2):372–379.
- Dawson A, Follett BK, Goldsmith AR, Nicholls TJ. Hypothalamic gonadotropin-releasing hormone and pituitary and plasma FSH and prolactin during photostimulation and photorefractoriness in intact and thyroidectomized starlings (*Sturnus vulgaris*). *J Endocrinol* 1985;105(1):71–77. [PubMed: 3921645]
- Dawson A, King VM, Bentley GE, Ball GF. Photoperiodic control of seasonality in birds. *J Biol Rhythms* 2001;16(4):365–380. [PubMed: 11506381]
- Duffy DL, Bentley GE, Drazen DL, Ball GF. Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. *Beh Ecol* 2000;11(6):654–662.
- Farner, D.; Donham, R.; Matt, K.; Mattocks, P.; Moore, M.; Wingfield, J. The nature of photorefractoriness. In: Mikami, S.; Homma, K.; Wada, M., editors. *Avian endocrinology: environmental and ecological perspectives*. Tokyo: Japanese Scientific Society Press; 1983. p. 149–166.
- Follett BK, Hinde RA, Steel E, Nicholls TJ. The influence of photoperiod on nest building, ovarian development and luteinizing hormone secretion in canaries (*Serinus canarius*). *J Endocrinol* 1973;59(1):151–162. [PubMed: 4748517]

- Fusani L, Van't Hof T, Hutchison JB, Gahr M. Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. *J Neurobiol* 2000;43(3):254–268. [PubMed: 10842238]
- Goldsmith AR, Ivings WE, Pearce-Kelly AS, Parry DM, Plowman G, Nicholls TJ, Follett BK. Photoperiodic control of the development of the LHRH neurosecretory system of European starlings (*Sturnus vulgaris*) during puberty and the onset of photorefractoriness. *J Endocrinol* 1989;22(1):255–268. [PubMed: 2671239]
- Hahn TP, Ball GF. Changes in brain GnRH associated with photorefractoriness in house sparrows (*Passer domesticus*). *Gen Comp Endocrinol* 1995;99(3):349–363. [PubMed: 8536947]
- Hahn, TP.; Boswell, J.; Wingfield, JC.; Ball, GF. Temporal flexibility in avian reproduction: Patterns and mechanisms. In: Nolan, V., Jr; Ketterson, E.; Thompson, C., editors. *Current Ornithology* 14. New York and London: Plenum; 1997. p. 39-80.
- Hahn TP, Pereyra ME, Sharbaugh SM, Bentley GE. Physiological responses to photoperiod in three cardueline finch species. *Gen Comp Endocrinol* 2004;137(1):99–108. [PubMed: 15094340]
- Hamner WM. The photorefractory period of the House Finch. *Ecology* 1968;49:211–227.
- Hinde RA. Temporal relations of brood patch development in domesticated canaries. *Ibis* 1962;104:90–97.
- Johnson F, Bottjer SW. Hormone-induced changes in identified cell populations of the higher vocal center in male canaries. *J Neurobiol* 1993;24(3):400–418. [PubMed: 8492114]
- Leitner S, Van't Hof TJ, Gahr M. Flexible reproduction in wild canaries is independent of photoperiod. *Gen Comp Endocrinol* 2003;130(2):102–108. [PubMed: 12568786]
- Leitner S, Voigt C, Garcia-Segura LM, Van't Hof T, Gahr M. Seasonal activation and inactivation of song motor memories in wild canaries is not reflected in neuroanatomical changes of forebrain song areas. *Hormones and Behavior* 2001;40(2):160–168. [PubMed: 11534977]
- Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F. A relationship between behavior, neurotrophin expression, and new neuron survival. *Proc Natl Acad Sci USA* 2000;97(15):8584–8589. [PubMed: 10890902]
- Macdougall-Shackleton SA, Katti M, Hahn TP. Tests of absolute photorefractoriness in four species of cardueline finch that differ in reproductive schedule. *J Exp Biol* 2006;209(Pt 19):3786–3794. [PubMed: 16985195]
- MacDougall-Shackleton SA, Deviche PJ, Crain RD, Ball GF, Hahn TP. Seasonal changes in brain GnRH immunoreactivity and song-control nuclei volumes in an opportunistically breeding songbird. *Brain Behav Evol* 2001;58(1):38–48. [PubMed: 11799277]
- Nicholls TJ, Goldsmith AR, Dawson A. Photorefractoriness in birds and comparison with mammals. *Physiol Rev* 1988;68(1):133–176. [PubMed: 3275941]
- Nottebohm F. Testosterone triggers growth of brain vocal control nuclei in adult female canaries. *Brain Res* 1980;189(2):429–436. [PubMed: 7370785]
- Nottebohm F. A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. *Science* 1981;214(4527):1368–1370. [PubMed: 7313697]
- Nottebohm F, Arnold AP. Sexual dimorphism in vocal control areas of the songbird brain. *Science* 1976;194(4261):211–213. [PubMed: 959852]
- Nottebohm F, Nottebohm ME, Crane L. Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. *Behav Neural Biol* 1986;46(3):445–471. [PubMed: 3814048]
- Pereyra ME, Sharbaugh SM, Hahn TP. Interspecific variation in photo-induced GnRH plasticity among nomadic cardueline finches. *Brain Behav Evol* 2005;66(1):35–49. [PubMed: 15821347]
- Sartor JJ, Ball GF. Social suppression of song is associated with a reduction in volume of a song-control nucleus in European starlings (*Sturnus vulgaris*). *Behav Neurosci* 2005;119(1):233–244. [PubMed: 15727528]
- Sartor JJ, Balthazart J, Ball GF. Coordinated and dissociated effects of testosterone on singing behavior and song control nuclei in canaries (*Serinus canaria*). *Horm Behav* 2005;47(4):467–476. [PubMed: 15777813]
- Steel E, Hinde R. Effect of artificially increased day length in winter on female domesticated Canaries. *J Zool Lond* 1966;149:1–11.

- Storey CR, Nicholls TJ. Some effects of manipulation of daily photoperiod on the rate of onset of a photorefractory state in canaries (*Serinus canarius*). *Gen Comp Endocrinol* 1976;30(2):204–208. [PubMed: 992342]
- Storey CR, Nicholls TJ. Failure of exogenous melatonin to influence the maintenance or dissipation of photorefractoriness in the canary, *Serinus canarius*. *Gen Comp Endocrinol* 1978;34(4):468–470. [PubMed: 648875]
- Tramontin AD, Brenowitz EA. Seasonal plasticity in the adult brain. *Trends Neurosci* 2000;23(6):251–258. [PubMed: 10838594]
- Tramontin AD, Perfito N, Wingfield JC, Brenowitz EA. Seasonal growth of song control nuclei precedes seasonal reproductive development in wild adult song sparrows. *Gen Comp Endocrinol* 2001;122(1):1–9. [PubMed: 11352547]
- Urbanski HF. Photoperiod and modulation of luteinizing hormone secretion in orchidectomized Syrian hamsters and the influence of excitatory amino acids. *Endocrin* 1992;131:1665–1669.
- Urbanski HF, Kim SO, Connolly ML. Influence of photoperiod and 6-methoxybenzoxazolinone on the reproductive axis of inbred LSH/Ss Lak male hamsters. *J Reprod Fertil* 1990;26:437–444.
- Voigt C, Leitner S. Breeding biology of the island canary *Serinus canaria* (AVES: Fringillidae) on the Desertas island Ilhéu Chão. *Bol Mus Mun Funchal* 1998;50:117–124.
- Voigt C, Goymann W, Leitner S. Green matters! Growing vegetation stimulates breeding under short-day conditions in wild canaries (*Serinus canaria*). *J Biol Rhythms* 2007;22(6):554–557. [PubMed: 18057330]
- Young KA, Ball GF, Nelson RJ. Photoperiod-induced testicular apoptosis in European starlings (*Sturnus vulgaris*). *Biol of Reprod* 2001;64:706–713. [PubMed: 11159376]
- Zar, JH. *Biostatistical Analysis*. Englewood Cliffs: Prentice-Hall, Inc.; 1974. p. 185-186.

Figure 1.

Changes in the mean value for gonadal size when maintained on a photoperiod of 14L:10D over the course of the experiment. The points represent the mean and the error bars represent $\pm$ the standard error of the mean (SE). (A) Male testis diameter in mm, significantly increase after photostimulation followed by a dramatic decline before 11 weeks post stimulation.

Follicle volume in female layers (B) and non-layers (C). Layers average includes only those birds exhibiting marked follicular development resulting in egg laying. Non-layers include all birds with moderate follicle development and exhibited brood patch development, but did not lay eggs.

Figure 2.

Mean male testosterone levels and gonad size at the time of brain collection. Blood was drawn prior to each laparotomy, as well as on the day the photostimulated group was returned to 14L:10D and 2 days prior to the day of brain collection. The 'Initial phototreatment' line indicates when all birds were on 14L:10D. After weeks 17 and 18 the graph reflects the average testosterone concentration for each treatment group. The stacked column denotes the average size of the male gonad in the second experiment for each treatment group at the time of brain collection. Bars represent $\pm$ SE. Asterisks indicate means among the treatment groups that differ significantly ($p < 0.05$).

Figure 3.

Bar graphs representing the results of the quantification of the GnRH immunoreactivity. The bars represent mean values and the lines within each bar are $\pm$ SE. Asterisks indicate means that differ significantly ($p < 0.05$) within a sex. Letters indicate significant difference between groups when sex is pooled. (A) Percentage POA area covered by GnRH immunoreactivity. (B) Number of cells immunoreactive for GnRH in the POA,

Figure 4.

Photomicrographs illustrating the pattern of GnRH immunoreactivity in the caudal POA in males collected in three different reproductive states. Low magnification shots are presented in panels A–C. High magnification shots presented in panels D–F correspond to the box drawn in the respective low magnification shot. Panels A and D are from a photorefractory birds, panels B and E are from a photosensitive bird and panels C and F are from a photostimulated bird. Bars indicate 500μm (A–C) and 100μm (D–F)

Figure 5.

Bar graphs illustrating the results of the quantification of key forebrain song nuclei in male and female Border canaries in three different reproductive states. The bars represent mean values of nuclei measured on one side of the brain; the lines within the bar represent $\pm$ SE. Asterisks indicate means that differ significantly ($p < 0.05$) within a sex. (A) HVC Volume. (B) RA volume. (C) Area X volume.

Figure 6.

Photomicrographs illustrating the boundaries of three forebrain song control based on Nissl stained material taken from male Border canaries in three physiological states. HVC is presented in panels A, D, and G. Area X is presented in panels B, E and H. RA is presented in panels C, F and I. Bars indicate 500 μ m.