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Effectiveness of the GnRH agonist Deslorelin as a tool to decrease levels of circulating testosterone in zebra finches

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ABSTRACT

Songbirds are widely used in studies of the neurobiology underlying learning, memory and performance of the sounds used in vocal communication. Development and activity of neurons in many brain sites implicated in those behaviors are closely related to levels of circulating testosterone. Approaches to understand the effects of testosterone in songbirds are presently limited to testosterone implants, which elevate testosterone levels to supraphysiological values, or castration, which eliminates gonadal production of testosterone. Previous studies in mammals indicate that GnRH agonists may be an effective tool to reduce testosterone within that range of extremes and without invasive surgery. To evaluate the effectiveness of the GnRH agonist Deslorelin as a tool to modulate levels of testosterone in songbirds, we recorded the effects of Deslorelin in adult male zebra finches. We recorded songs, body mass and blood testosterone levels pre-treatment, then we gave each bird a small subcutaneous implant of Deslorelin. We measured blood plasma testosterone levels weekly and recorded song behavior and gross morphology of brain, testes and heart at the end of each experiment. Testosterone levels were reduced at the 5 mg/kg dose, and the very slight song changes we observed at that dose were like those reported for castrated zebra finches. As expected, there were no changes in the number of cells in androgen-sensitive brain structures. Suppression of testosterone at the 5 mg/kg dose was reversible through implant removal. Thus, Deslorelin is a new tool to transiently suppress testosterone levels without the invasiveness and undesirable aftereffects of surgical castration.

1. Introduction

Songbirds are an excellent animal model for investigating the relation between neurophysiology, hormones, and behavior. At a very young age, male songbirds memorize song models then recall them during development and rely on auditory feedback to refine their imitation, often arriving at performances nearly identical to the model. Throughout that impressive feat of learning and memory, a set of specialized neural circuits also undergoes maturation and refinement (Nick and Konishi, 2005; Roberts et al., 2010; Volman, 1993). Male songbirds possess a dedicated network of brain sites, collectively called the "song system", that are essential for learning and performing songs (Mooney et al., 2008). Many of those song system neurons express hormone receptors through which testosterone and its metabolites can influence neural activity, the properties of the associated songs, and other aspects of male courtship behavior (Ball et al., 2004; Brenowitz, 2004; London et al., 2009; Schlinger and Brenowitz, 2009).

Reducing the level of circulating testosterone in adult zebra finches using castration or increasing that level using testosterone implants can alter the biophysical properties and activity of neurons in the song system (Wang et al., 2014; White et al., 1999). Elevation of testosterone levels can also impair song learning in juvenile zebra finches, leading to early acquisition of adult-like stereotypy and atypical structure of individual syllables and song syntax (Alliende et al., 2010; Korsia and Bottjer, 1991). The effects of testosterone have been studied most extensively in zebra finches, but the importance of the relation between hormones and behavior is especially apparent in seasonally-breeding songbirds, as song-related brain sites can dramatically increase in volume and cell number in response to elevated testosterone levels that emerge with lengthening photoperiod (Ball et al., 2004; Brenowitz, 2004). Thus, zebra finches and other species of songbirds provide a rich context in which to study the effects of hormones, specifically testosterone, on brain and behavior.

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Understanding the role of testosterone in shaping brain and behavior requires experimental manipulation of hormone levels. Effects of testosterone and its metabolites can be manipulated using tools such as aromatase inhibitors or receptor antagonists, however songbird neurobiologists are currently rather limited in approaches to alter levels of circulating testosterone. Testosterone levels can be elevated using testosterone implants, or gonadal production of testosterone can be eliminated through castration. Castration is effective but also very invasive, commonly resulting in reduction or elimination of singing while the bird recovers, and it is irreversible, preventing experimenters from being able to restore endogenous production following a period of reduced testosterone. The goal of this study was to develop a minimally invasive and reversible way of reducing levels of circulating testosterone.

Deslorelin (tradename: Suprelorin, PepTech Corporation) is a subcutaneous implant that slowly releases a synthetic agonist of gonadotropin-releasing hormone (GnRH) over the course of many months (Trigg et al., 2001, 2006). Deslorelin affects the anterior pituitary, activating gonadotropic cells in the hypothalamic–pituitary–gonadal (HPG) axis. Those cells release leutinizing hormone (LH) and follicle stimulating hormone (FSH) that enter the bloodstream and induce testicular production of testosterone (Junaidi et al., 2009; Trigg et al., 2001). In studies of the effects of Deslorelin in mammals, initial exposure activates the HPG pathway and commonly causes an upwards surge, or "flare", in the level of circulating testosterone (Ponglowhapan, 2011). With continuous activation, as occurs in the case of an implant, GnRH receptors on gonadotropic cells become downregulated, causing those cells to reduce or cease their release of LH and FSH, and in turn causing a decrease in testicular testosterone production (Conn and Crowley, 1994; Horvath et al., 2002). The structure and function of the HPG axis are strongly conserved across mammals and birds (Ottinger and Bakst, 1995; Ottinger et al., 2002), and Deslorelin has been used safely in chickens (*Gallus gallus domesticus*), pigeons (*Columba livia domestica*) and Japanese quail (*Coturnix japonica*) to manipulate aspects of endocrine and reproductive status other than testosterone levels (e.g., levels of LH and androstenedione) (Cowan et al., 2014; Nooan et al., 2012; Petritz et al., 2013). These data led us to hypothesize that Deslorelin will also be a safe and effective tool to decrease levels of circulating testosterone in songbirds.

We investigated the degree to which Deslorelin suppresses levels of circulating testosterone in adult male zebra finches (*Taeniopygia guttata*). Deslorelin had no deleterious effects on health or body weight, but testosterone levels were significantly reduced in birds that received a 5 mg/kg dose. To investigate whether changes in testosterone were associated with changes in morphology, we measured the testes and heart at the conclusion of each experiment (Edwards et al., 2013; Skinner et al., 2009). We also investigated the degree to which Deslorelin affected brain and behavior by comparing songs recorded from each male before and after the implant and measuring cell density of sites in the song system. Finally, we removed the implant in a subset of birds to determine the time course over which removal of Deslorelin could restore plasma testosterone levels to their pre-implant values.

2. Materials and methods

2.1. Care and housing of subjects

We housed adult male zebra finches ($N = 53$, age > 120 days post hatch) in same-sex cages in our colony throughout the study (groups of 8–12 birds housed together in 8 ft^3 cages in which they were in constant visual and acoustic contact with all other members of the colony; 15:9 light:dark cycle). Seed and water were available *ad libitum*, enabling us to avoid the variance in testosterone levels and zebra finch song behavior that can emerge with fasting (Lynn et al., 2010). All experimental procedures were in compliance with the University of Wyoming Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A-3216-01) and all state and federal regulations for the housing and use of songbirds.

2.2. Experimental groups and placement of Deslorelin implants

We separated birds into 4 groups that each received a different treatment: (1) a low dose group (1 mg of Deslorelin per kg of body weight, $N = 20$ birds), (2) a high dose group (5 mg/kg, $N = 21$ birds), (3) a group that received the high dose, had the implant removed after 4 weeks and then was monitored weekly for an additional 7 weeks ($N = 4$ birds; we did not perform a sham removal in the birds that received a 1 or 5 mg/kg dose and were monitored beyond 4 weeks), and (4) a control group ($N = 8$ birds). In birds that received an implant, we locally anesthetized the skin overlying the abdomen, made a small incision, placed the implant and closed the skin using veterinary adhesive (VetBond). Even the high dose implants were quite small (cubes of approximately 2 mm on each side), and birds did not peck or otherwise agitate the site of implantation. We left the implants in place for 1, 2, 4 or 8 weeks to investigate the time course over which Deslorelin had its effects ($N = 5$ or 6 birds in each group at each dose, hence the $N = 20$ and 21 for low and high doses, respectively; detailed in Fig. 1). Controls did not receive an implant but were housed and handled in the same way as their experimental counterparts. We monitored birds daily, and we weighed each bird at the start of the study and then each week thereafter to determine if Deslorelin caused any changes in body weight or overall health (e.g., survival, vigor, absence of puffed feathers).

2.3. Blood collection and measurement of circulating testosterone levels

We collected blood samples from the brachial vein of either wing of each bird each week. Blood collection began as early as 1 h following lights-on and as late as 7 h following lights on. We used a sterile cotton swab to apply a small amount of sterile heparin (sodium injection) to the skin overlying that site. We pricked the skin and vein using a sterile stainless steel needle (25G) and collected the blood using a heparinized capillary tube (250 μl, Kimble Chase). We began collecting blood within 1 min of taking the bird into our hands, and it typically took 1–2 min to collect blood into the capillary tube. We typically collected approximately 120 μl of blood in each sample, consistent with procedures used by others (Deregnaucourt et al., 2013; Rutkowska et al., 2005) and recommended practices regarding blood collection from small songbirds (Owen, 2011). Immediately after collection, we centrifuged the sample (5000G, 5 min, 4 C), removed the plasma without disturbing the pellet (typically approximately 60 μl per sample), placed the plasma into another sterile microcentrifuge tube, and froze the sample (−20 C). We measured the amount of testosterone in each sample using an enzyme immunoassay kit (Salimetrics; cross-reactivity: 36.4% for dihydrotestosterone, 21.0% for 19-nortestosterone, 1.9% for 11-hydroxytestosterone, 1.2% for androstenedione, <1% for all other compounds tested; validated using the same assay as Remage-Healey et al. (2008), correlation coefficient = 0.76). In each sample, the amount of testosterone was measured in duplicate (25 μl of blood plasma in each of 2 wells in the kit). The kit included a set of standards ranging from 6.1 to 600 pg/ml and had a minimal detection limit of 1 pg/ml. To ensure that we did not approach the limit of our measurement kit and to eliminate the need to extrapolate from our

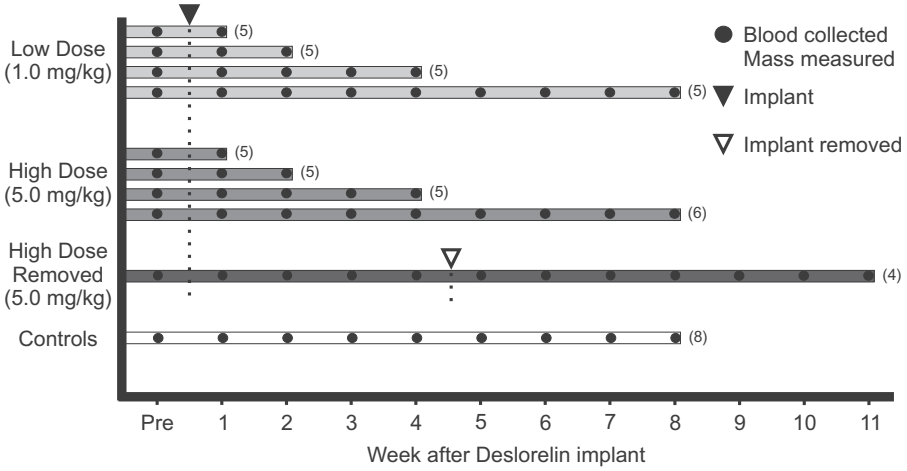

Fig. 1. Birds were divided into control, low dose and high dose groups (time of the Deslorelin implant is indicated by a filled triangle). Blood was collected and body mass was measured at the start of the study and every week thereafter (filled circles). Tissue was collected from each bird at the end of an observation period of 1, 2, 4 or 8 weeks (horizontal bars). In another group of birds (high dose removed), we removed the high dose implant after 4 weeks (open triangle) and continued to collect blood and measure body mass for an additional 7 weeks. Controls received no implant but were housed and handled in the same way as their experimental counterparts. Numbers in parentheses indicate the number of birds in each group.

standards, any measured value that fell below 6.1 pg/ml was entered into the dataset as 6.1 pg/ml. That affected only a small number of testosterone measurements (15 of 366 measurements, 4.1%) and if anything caused us to slightly underestimate the efficacy of Deslorelin as a suppressor of endogenous testosterone.

2.4. Song recording and analysis

We recorded the songs performed by each bird at the start of the study (minimum of 10 songs collected for each bird; recorded 2–7 days prior to Deslorelin implant) and immediately before the end of the study (recorded 4 days prior to sacrifice). We recorded each bird individually in sound attenuating chambers with a microphone (Shure SM57) adjacent to the bird's cage (41 cm × 30 cm × 24 cm; birds that resided in the chamber overnight were exposed to the same 15:9 light:dark cycle as in the colony). We monitored birds continuously and saved song files onto a computer hard drive using custom software (Sound Analysis Pro) (Tchernichovski et al., 2000); songs were bandpass filtered 300–10,000 Hz). In offline analysis, we quantified the spectral and temporal properties of each song (Sound Analysis Pro and custom Matlab software).

2.5. Tissue collection and measurement

At the conclusion of the observation period for each bird (1, 2, 4 or 8 weeks, detailed above), we euthanized the bird using an overdose of isoflurane. We perfused the bird transcardially with physiological saline followed by 4% paraformaldehyde, collected the brain, heart and testes, and cryoprotected all tissues in 30% sucrose in 4% paraformaldehyde. After fixation for at least 48 h, we dabbed the tissue dry and measured the length, width and mass of the heart and testes (digital scale with 0.1 mg resolution and digital calipers with 0.01 mm resolution).

We investigated song-related brain nuclei by sectioning each hemisphere (50 micron parasagittal sections), mounting sections onto glass slides, and staining all nuclei using a coverslipping solution containing fluorescent 4′,6-diamidino-2-phenylindole, Dihydrochloride (DAPI; ProLong Gold AntiFade Reagent, Life Technologies). We imaged sections at 20X (fluorescent microscope; image size 500 microns × 500 microns), and we quantified the number of cells present in sections of brain sites in the song system in each hemisphere of each bird (brain sites were HVC, RA and Area X; HVC: abbreviation used as a proper noun; RA: robust nucleus of the arcopallium; Area X: Area X of the medial striatum). For each brain region, we analyzed 2 brain sections: one that lay in the medial half of the structure (one third of the way along the full medial–lateral extent) and another that lay in the lateral half of the structure (two thirds of the full medial–lateral extent). The size of the area sampled was the same in each image, and we counted the number of cells present in that standardized area (Matlab Image Processing Toolbox). To minimize the influence of cells that lay primarily outside of the plane of section, we enforced the criterion that the area of each cell had to be at least 60 microns^2 in order to be included in our analysis. We quantified each of the two sections collected from each structure in each bird and represented that structure by the average of those two values.

2.6. Statistical analyses

We used one-way ANOVAs and Tukey's HSD post hoc tests to compare measurements of body weight, testosterone levels, brain cell counts and properties of song behavior. In our comparisons of the mass and dimension of the heart and testes, we used two-way ANOVAs and post hoc Tukey's HSD post hoc tests. All tests had an alpha level of 0.05 as the measure of statistical significance, and all results are expressed as mean ± standard error.

3. Results

3.1. Deslorelin reduced levels of circulating testosterone

The birds in each of the control (N = 8 birds), low dose (N = 20) and high dose groups (N = 21) were indistinguishable in their levels of blood plasma testosterone at the start of the study (one-way ANOVA, $F(2, 48) = 0.66$, $p = 0.52$, Fig. 2A). Pre-treatment levels of testosterone ranged from 7.65 to 560.02 pg/ml (mean ± SE: 98.5 ± 15.8, N = 49 birds), comparable with some previous measurements of testosterone levels in male zebra finches (e.g., 161 ± 11 pg/ml in Remage-Healey et al., 2008 and 170 ± 36 pg/ml in Korsia and Bottjer, 1991) and lower than some others (Koren et al., 2012; White et al., 1999). Among control birds, testosterone levels varied across the duration of the study, but there were no

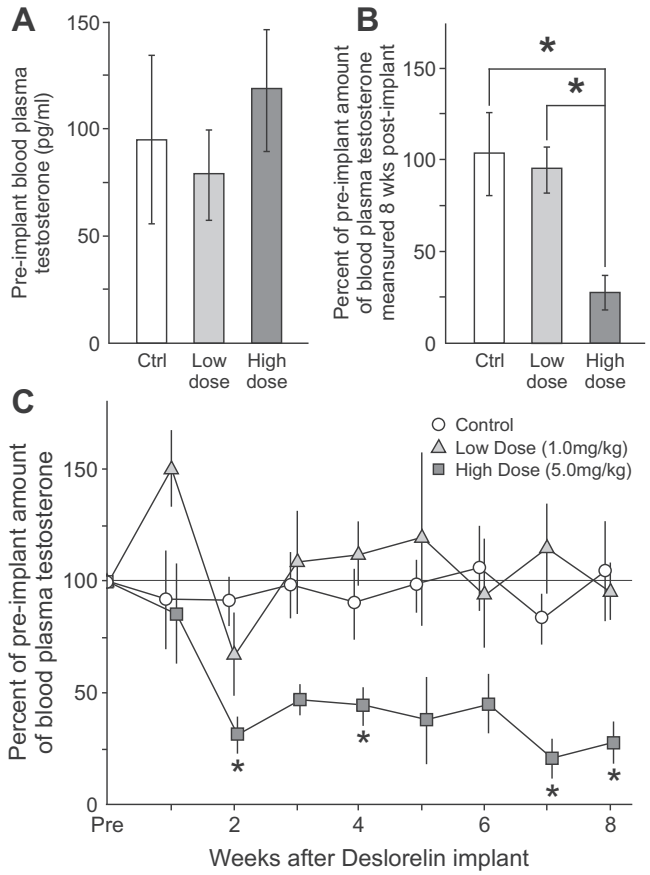

Fig. 2. (A) Groups of birds were indistinguishable in their levels of blood plasma testosterone at the start of the study. (B) By the end of the study, testosterone was significantly suppressed in the high dose group but not the low dose group or controls. (C) A trend toward a flare of testosterone was evident in week 1 for the low dose group, but there were no weeks in which the low dose induced testosterone levels that were significantly different than controls. In contrast, testosterone levels were suppressed by 2 weeks post-implant in the high dose group and remained suppressed throughout the 8 weeks of observation. All points and error bars indicate mean ± SE.

significant changes in testosterone levels over time (one-way ANOVA, $F(8, 58) = 0.19$, $p = 0.99$, $N = 8$ weeks and sample sizes of 8, 8, 8, 8, 8, 3, 3, 6, 7 birds from the start of the study through week 8, Fig. 2B and C).

In contrast to control birds, testosterone levels varied across time in birds that received Deslorelin. Among birds that received the low dose of 1 mg/kg, there was significant variation in the amount of testosterone measured each week (one-way ANOVA, $F(8, 89) = 2.19$, $p = 0.04$, $N = 8$ weeks and samples sizes of 20, 20, 15, 10, 10, 5, 5, 5, 5 birds from the start of the study through week 8, Fig. 2B and C). Post-hoc testing revealed a significant difference between weeks 1 and 2, influenced by a flare of testosterone in some birds in which levels measured at week 1 were higher than their respective pre-implant values (15 of 20 birds had week 1 testosterone levels that were greater than that measured prior to implant; compare with 4 of 8 birds in the control group and 5 of 21 birds in the high dose group). Apart from week 1, however, there were no weeks in which a low dose of Deslorelin induced testosterone levels that were significantly different than levels detected in control birds (Tukey's HSD, $p > 0.27$ in all cases).

Among birds that received the high dose of 5 mg/kg, suppression of testosterone was much more apparent (one-way ANOVA, $F(8, 92) = 3.67$, $p = 0.001$, $N = 8$ weeks and samples sizes of 21,21,16,11,10,5,5,5,5 birds from the start of the study through week 8). By the end of the study, testosterone levels were 27.7 ± 9.2% of their pre-implant values, significantly lower than in control birds (104.2 ± 21.9%, Tukey's HSD, $p = 0.02$) or in birds that received the low dose of Deslorelin (95.3 ± 12.2%, Tukey's HSD, $p = 0.05$, Fig. 2B). Suppression of testosterone levels was evident by week 2 (Tukey's HSD, $p = 0.02$) and persisted throughout the rest of the study (significant differences from control were also evident in weeks 4, 7 and 8; p-values of 0.04, 0.02 and 0.02, respectively; asterisk symbols in Fig. 2C). Therefore, 5 mg/kg is an adequate dose to suppress testosterone production in adult male zebra fiches.

3.2. Deslorelin did not affect health, body weight or gross morphology

In no case did we observe signs of distress that were in any way related to the implant procedure (e.g., lethargy, puffed feathers, infected site of Deslorelin implant, etc.). Furthermore, there were no differences in body weight among birds in the control ($N = 8$), low dose ($N = 20$) and high dose ($N = 21$) groups at week 8 (one-way ANOVA, $F(2, 12) = 0.87$, $p = 0.44$) or over the course of the study (one-way ANOVA, $F(2, 48) = 0\ 46$, $p = 0.63$). There were also no differences in the mass or dimension of the testes or heart (summarized in Tables 1 and 2). Together these data reveal that a subcutaneous implant of Deslorelin is safe for use in zebra finches over the durations tested here.

3.3. Deslorelin had little or no effect on song properties or cell numbers in the song system

Neither the high dose nor the low dose of Deslorelin had a significant effect on the number of songs that each male performed (one-way ANOVA, $F(3, 11) = 0.18$, $p = 0.91$). Although these groups were not different, there was nonetheless a trend toward fewer songs performed by birds that received greater amounts of Deslorelin (songs performed per day: control group = 86.5 ± 42.5; low dose = 67.2 ± 13.3; high dose = 49.3 ± 31.1), which is consistent with decreased song production following castration (Arnold, 1975). In addition, there was little or no change in the spectral properties of individual notes or the temporal properties of note sequence and song tempo, which is also consistent with the lack of change in song properties following castration (Arnold, 1975). Among the 17 spectral and temporal properties

Table 1
Gross morphology of testes and heart as a function of Deslorelin treatment.

Variable	Treatment groups			One-way ANOVA	
	Control	Low	High	F	p-value
Left testis mass (g)	0.02 ± 0.002	0.02 ± 0.001	0.02 ± 0.001	$F(14, 32) = 2.07$	0.14
Left testis volume (mm^3)	112.78 ± 15.16	83.88 ± 5.63	87.11 ± 8.95	$F(14, 32) = 0.09$	0.91
Right testis mass (g)	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.001	$F(14, 32) = 2.38$	0.11
Right testis volume (mm^3)	66.08 ± 12.06	59.50 ± 6.11	56.20 ± 5.43	$F(14, 32) = 0.38$	0.69
Heart mass (g)	0.26 ± 0.02	0.27 ± 0.01	0.21 ± 0.01	$F(14, 32) = 1.07$	0.35

There were no significant differences in the mass or volume of the testes or heart as a function of Deslorelin treatment.

Table 2
Gross morphology of testes and heart throughout the exposure to Deslorelin.

Variable	Weeks after Deslorelin implant (values are pct. change from start of experiment)					One-way ANOVA	
	Pre-implant	Week 1	Week 2	Week 4	Week 8	*F*	*p*-value
Left testis mass (g)	0.02 ± 0.002	0.02 ± 0.001	0.01 ± 0.001	0.02 ± 0.002	0.02 ± 0.002	$F(14, 33) = 0.09$	0.98
Left testis volume (mm^3)	112.78 ± 15.16	77.50 ± 8.62	68.97 ± 7.09	105.49 ± 14.54	90.01 ± 7.30	$F(14, 33) = 0.68$	0.61
Right testis mass (g)	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.001	$F(14, 33) = 1.87$	0.14
Right testis volume (mm^3)	66.08 ± 12.06	58.11 ± 11.17	42.40 ± 4.39	65.00 ± 7.81	65.88 ± 6.22	$F(14, 33) = 0.77$	0.55
Heart mass (g)	0.26 ± 0.02	0.23 ± 0.01	0.24 ± 0.02	0.26 ± 0.01	0.24 ± 0.01	$F(14, 33) = 1.64$	0.19

There were no significant differences in the mass or volume of the testes or heart as a function of time following the Deslorelin implant.

Table 3
Spectral and temporal properties of song behavior.

	Treatment groups (values are pct. change from start of experiment)			One-way ANOVA	
	Control	Low	High	*F*	*p*-value
Note duration (s)	0.01 ± 0.02	0.02 ± 0.03	0.04 ± 0.02	$F(3, 36) = 0.23$	0.88
Fundamental frequency (Hz)	0.01 ± 0.03	0.05 ± 0.04	0.13 ± 0.09	$F(2, 72) = 1.35$	0.27
Frequency modulation	0.01 ± 0.02	−0.03 ± 0.03	0.01 ± 0.03	$F(2, 72) = 0.85$	0.43
Spectral entropy	0.02 ± 0.02	−0.08 ± 0.04	0.13 ± 0.06	$F(2, 72) = 6.07$	0.004*
Frequency (Hz)	0.01 ± 0.02	−0.03 ± 0.01	−0.05 ± 0.02	$F(2, 72) = 2.43$	0.10
Amplitude modulation 2 ($1/t$)	0.08 ± 0.04	−0.02 ± 0.04	−0.08 ± 0.05	$F(2, 72) = 3.44$	0.04*
Amplitude (dB)	0.02 ± 0.01	−0.01 ± 0.01	−0.01 ± 0.01	$F(2, 72) = 3.83$	0.03*
Tempo median (notes/s)	0.02 ± 0.06	0.03 ± 0.02	0.13 ± 0.07	$F(2, 11) = 0.94$	0.42
Tempo minimum (notes/s)	0.03 ± 0.07	0.01 ± 0.01	−0.01 ± 0.07	$F(2, 11) = 0.14$	0.87
Tempo maximum (notes/s)	−0.01 ± 0.08	0.20 ± 0.13	0.06 ± 0.11	$F(2, 11) = 1.09$	0.37
Tempo mean (notes/s)	−0.02 ± 0.06	0.05 ± 0.06	0.05 ± 0.04	$F(2, 11) = 0.65$	0.54
Sequence linearity	0.01 ± 0.03	−0.02 ± 0.02	−0.12 ± 0.10	$F(2, 11) = 1.56$	0.25
Sequence consistency	−0.01 ± 0.02	−0.01 ± 0.02	−0.03 ± 0.08	$F(2, 11) = 0.14$	0.87
Sequence entropy	−0.01 ± 0.03	0.25 ± 0.47	0.01 ± 0.01	$F(2, 11) = 0.27$	0.77
Number of epochs of note repetition	0.89 ± 1.15	−0.01 ± 0.22	3.90 ± 2.13	$F(3, 9) = 1.62$	0.25
Average number of repeated notes in each epoch	0.75 ± 0.91	0.05 ± 0.25	1.01 ± 0.39	$F(3, 9) = 0.34$	0.79
Percent of song duration comprising note repetition	0.01 ± 0.23	0.09 ± 0.29	0.67 ± 0.45	$F(3, 9) = 0.72$	0.56

Among the 17 song properties that we measured, Deslorelin was associated with changes in only 3 properties. A high dose was associated with a significant decrease in amplitude modulation, and a low dose was associated with a decrease in song amplitude ($p < 0.05$ in one-way ANOVA and $p < 0.05$ in Tukey's post hoc HSD comparison vs. control birds). A difference in spectral entropy was evident between the high dose and the low dose groups ($p < 0.05$ in one-way ANOVA and $p < 0.05$ in Tukey's post hoc HSD comparison of high dose vs. low dose birds), but neither group was different than controls. Cases of significance are indicated by an asterisk.

that we quantified for songs produced at the beginning and the end of the study, there were minor changes in song amplitude in some birds (5 mg/kg dose vs. controls) and changes in amplitude modulation in others (1 mg/kg dose vs. controls). We also noted a difference in spectral entropy between low-dose and high-dose groups, but neither was different than controls (Table 3). Therefore, there was little impact of Deslorelin and reduced testosterone on zebra finch song performance. Consistent with that finding, there were also no differences in the numbers of cells present in androgen-sensitive forebrain areas, including HVC, RA and Area X (Table 4; there were also no treatment-related differences when the numbers of cells observed in each hemisphere were combined to describe each bird; one-way ANOVA tests, $p = 0.14$, 0.19 and 0.38 for HVC, RA and Area X, respectively).

3.4. Deslorelin-induced suppression of testosterone is reversible

Removal of the high dose implant of Deslorelin 4 weeks after implantation resulted in a rebound of testosterone levels from their suppressed state to supranormal levels ($N = 4$ out of 4 birds, Fig. 3). As with our other set of high-dose birds, there was significant variation in the amounts of testosterone detected over the course of the study, and that was true even if the bird that responded especially strongly (1648% at week 6, increased from an initial value of 16.1 pg/ml) was excluded from consideration (one-way ANOVA, $F(11, 35) = 7.36$, $p < 0.001$, $N = 3$ birds). Testosterone levels in the high-dose removed birds ($N = 4$) were significantly suppressed by week 2 (Tukey's post hoc, control vs. hi-dose removed, $p < 0.01$) and remained suppressed until we removed the implant (Tukey's post hoc, control vs. high dose removed, $p = 0.01$ and $p = 0.03$ in weeks 3 and 4, respectively). Within 1 week of removing the implant, testosterone levels went from 28.4 ± 8.2% of pre-implant levels to 387.3 ± 201.6% ($N = 4$ birds), which was a significant deviation from the persistent suppression at week 5 in birds that did not have their high dose implant removed (Tukey's post hoc, high dose removed vs. high dose, $p = 0.04$). This difference between birds that received a high dose versus birds that received a high dose then had it removed persisted in weeks 6 and 7 (Tukey's post hoc, $p < 0.01$ in both cases) but was no longer evident by week 8 (Tukey's post hoc, $p = 0.09$; Fig. 3). We could not continue that comparison in week 9 and beyond because we did not track our high dose birds beyond week 8 post-implant, however testosterone levels in birds that had the high dose removed at week 4 returned to approximately control levels in weeks 9 through 11 (118%, 116% and 99% of control values, respectively). Therefore, after 4 weeks of exposure to Deslorelin, removal of the implant caused testosterone values to surge to supranormal values for 3 weeks then return to normal in the following week and remain at approximately pre-implant levels thereafter.

4. Discussion

A 5 mg/kg dose of Deslorelin is an effective tool to decrease levels of circulating testosterone in adult male zebra finches. That dose decreased testosterone to approximately 35% of pre-implant

Table 4
Number of cells in androgen-sensitive brain areas.

Nucleus	Hemisphere	Treatment groups			One-way ANOVA	
		Control	Low	High	*F*	*p*-value
HVC	Left	106 ± 10.96	91.2 ± 6.45	114.7 ± 5.65	$F(2, 12) = 2.19$	0.16
HVC	Right	88.8 ± 13.01	110.9 ± 13.21	119.7 ± 9.16	$F(2, 12) = 1.78$	0.21
RA	Left	75.9 ± 10.45	65.4 ± 6.54	72.5 ± 3.36	$F(2, 12) = 0.53$	0.60
RA	Right	72.3 ± 7.28	71.6 ± 3.81	89 ± 3.70	$F(2, 12) = 3.58$	0.06
Area X	Left	120 ± 16.64	110.2 ± 4.93	114.9 ± 8.75	$F(2, 12) = 0.21$	0.21
Area X	Right	97 ± 9.90	122.5 ± 12.64	133.3 ± 9.64	$F(2, 12) = 2.97$	0.09

Neither dose of Deslorelin significantly affected the number of cells in HVC, RA or Area X of either hemisphere.

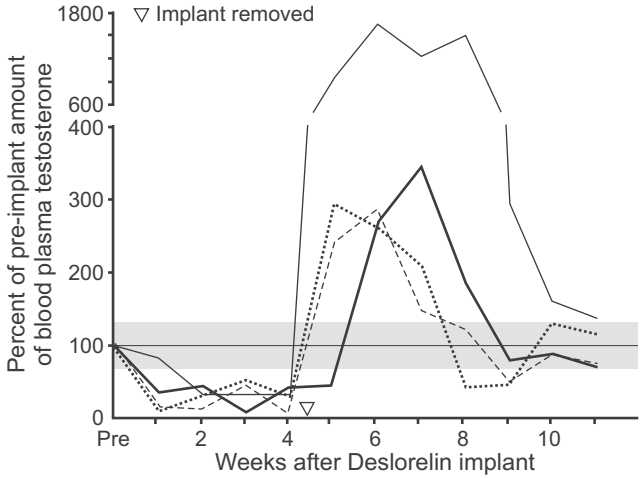

Fig. 3. Suppression of testosterone in response to a high dose of Deslorelin could be reversed by removal of the implant (open triangle at week 4). In all 4 birds that we tested (individual birds are indicated by different lines), testosterone levels rebounded to supranormal levels by week 6. Those levels remained elevated until week 8, then they returned to levels that were indistinguishable from controls (gray bar indicates the range of mean ± SE values observed in the control condition) and remained at approximately pre-implant levels thereafter.

levels, and suppression persisted throughout the remainder of the study. Suppression could be reversed by removing the implant, resulting in supranormal amounts of testosterone for 3 weeks post-removal then returning to control levels. Over the 8 weeks that we tracked the effects of Deslorelin, subcutaneous implants and the associated changes in testosterone had no adverse effects on the birds' overall health, the morphology of the heart or testes, or temporal properties of song. Consistent with the previous finding that castration reduces the number of songs performed by adult male zebra finches (Arnold, 1975), we observed a nonsignificant trend toward fewer songs performed by birds that received high doses of Deslorelin. There was little or no change in spectral properties of song, and we detected no changes in the number of cells present in androgen-sensitive regions in the song system (HVC, RA and Area X). These findings are consistent with previous observations that castration and the associated reduction of testosterone have little or no effect on the structure of adult zebra finch song or the number of cells present in those brain regions (Arnold, 1975, 1980; Bottjer and Hewer, 1992). These similarities between the effects of Deslorelin and the effects of castration reveal that Deslorelin is a new, minimally invasive and reversible alternative to surgical castration as a means of reducing levels of circulating testosterone in songbirds.

We tested the effects of Deslorelin in zebra finches because the majority of studies measuring the role of hormones in shaping song learning and performance have been performed using zebra finches, and that provided us a context in which to compare Deslorelin to the effects of castration. Testosterone has profound effects on song learning and development in juvenile zebra finches, just as testosterone has been shown to influence acquisition of speech in young children (Bottjer and Hewer, 1992; Korsia and Bottjer, 1991; White et al., 1999; Whitehouse et al., 2012). This influence may be mediated by testosterone either directly or indirectly through its aromatization into estradiol (Balthazart et al., 1990). These hormones affect song-related activity in the zebra finch brain (Ball et al., 2004), and they are produced by cells in the brain (London et al., 2009). Tools that impact the production of testosterone through the GnRH pathway (Kumar and Sharma, 2014) may affect song and the activity of the associated neurons by influencing neural activity through androgen receptors, estrogen receptors or both.

Testosterone plays a lesser role in maintaining song quality in adult zebra finches (Bottjer and Hewer, 1992), however the effects of testosterone and its metabolites are much greater in adults of other songbird species. For example, many songbird species undergo annual loss and replacement of large numbers of neurons in the song system, and that process is strongly dependent on surges in hormone levels that occur at the start of the breeding season. In Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*), the forebrain nucleus HVC expands dramatically during the transition from winter into the breeding season. Strikingly, when sparrows are moved from a short-day to a long-day photoperiod and given an implant of testosterone, HVC expands by 70% and approximately 50,000 new neurons are inserted into the HVC microcircuit in approximately one week (Tramontin et al., 2000). Cell loss and atrophy are similarly rapid when photoperiod is artificially reverted to winter status and testosterone implants are removed (Thompson et al., 2007). Additional results indicate that testosterone acts through neurotrophic factors such as BDNF to promote the generation and survival of new neurons in the adult songbird brain (Rasika et al., 1999). These data emphasize the utility of seasonal songbird species as a model in which to study how neuroendocrine and neurotrophic factors shape learned behavior and the structure and function of the underlying nervous system. We expect that Deslorelin will be especially useful as a tool to grade testosterone levels in experiments involving those species.

Testosterone levels surged in the first week after implanting the 1 mg/kg dose but not after implanting the 5 mg/kg dose. This could be due to individual differences in sensitivity to Deslorelin, but given the similarity of body mass and initial testosterone levels among our treatment groups, it seems unlikely that birds in one group were systematically different in their sensitivity. A flare in testosterone for the low dose but not the high dose could have arisen because of differences in the amount of Deslorelin in the birds' blood immediately following implantation. In mammals, the level of circulating Deslorelin is higher immediately following implantation than the plateau that occurs days later (Kraeling et al., 2000). Similar dynamics could account for why we observed a flare in week 1 after the 1 mg/kg dose followed by no systematic deviation from control levels in the following weeks. The absence of a flare in birds that received the 5 mg/kg dose could also have

been due to the fact that we measured testosterone levels after one week rather than a finer scale such as daily measurements. The flare may have occurred and testosterone levels had begun to be downregulated by one week after implantation. A future goal will be to further characterize the dose-dependence and the time course of the onset and offset of Deslorelin's effects. That higher-resolution understanding of the effects of Deslorelin will be informative for researchers seeking to use this tool in their studies of hormones and behavior.

Neither the 1 mg/kg nor the 5 mg/kg dose of Deslorelin affected testes weight or dimension. Studies in mammals reveal that suppression of testosterone production is associated with atrophy of the testes in some species, such as rats, but not in other species, such as mice (discussed in Edwards et al., 2013). In that light, it is perhaps not surprising that we saw no change in the properties of zebra finch testes even when testosterone production was significantly affected. Two factors could have played important roles in the observed lack of changes in the testes. First, the partial suppression of testosterone that we observed is of a lesser magnitude and a shorter duration than the near-complete elimination of testosterone reported in many studies of mammals that have had Deslorelin implanted for many months (Edwards et al., 2013). It remains to be seen whether the testes may become atrophied with very high doses and/or long-term administration of Deslorelin in zebra finches. Second, the lack of testicular atrophy in our dataset may reflect differences in the molecular composition of the testes of zebra finches versus species in which the testes do atrophy in association with suppressed gonadal production of testosterone. Because a lack of atrophy is also evident in some mammals, it is unlikely that this difference reflects some aspect of avian versus mammalian HPG axes. Instead, the presence or absence of testicular atrophy with suppression of testosterone production likely reflects species-level differences in how GnRH affects HPG function and the regulation of gonadal production of testosterone. Our findings extend the effects of Deslorelin to zebra finches, providing an additional species for comparative study of how those processes are regulated.

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