Received: 11 April 2024 | Revised: 29 June 2024 | Accepted: 4 September 2024

DOI: 10.1111/jne.13449

REVIEW ARTICLE

Journal of Neuroendocrinology WILEY

Photoperiodism, testosterone and adult neurogenesis in canaries (*Serinus canaria*)

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Funding information
National Institute of Neurological Disorders and Stroke, Grant/Award Number: RO1NS104008

Abstract

Domestic strains of canaries (*Serinus canaria*) variably respond to photoperiod changes and apparently stay in breeding state for extended periods. Fife Fancy canaries are supposed to be similar to the native species living at 27–39° north where photoperiod significantly changes across the year. Our birds showed reproductive cycles when exposed to light regimes mimicking the annual cycle of photoperiod. However after 6 months in short days (SD: 8L:16D), males developed large testes, as observed by X-ray tomography, and intense singing. Switching to long days (LD: 16L:8D) did not further increase song rate nor testes size but increased song duration, number of syllables per song, and trill occurrence frequency. No sign of regression was observed after 12 weeks in LD but return to SD produced a rapid decrease in testes size and singing activity below values in birds maintained throughout in SD. Fife Fancy thus does not seem to develop absolute but only relative refractoriness. The relatively high singing activity expressed by SD-photosensitive males does not seem to depend on high testosterone (T) concentrations. Singing did not correlate with plasma testosterone (T). Treatment with ATD + Flutamide only marginally decreased song rate and did not affect song quality nor song control nuclei volume. These birds are either supersensitive to low T levels or their reproductive physiology is activated by other mechanisms. Neurogenesis is increased by T and by LD but the function of new neurons incorporated in HVC is poorly understood. We developed a procedure based on X-ray focal irradiation to deplete neural progenitors adjacent to HVC and study the functional consequences. The decrease in neurogenesis increased the variability of T-induced songs in females and decreased their bandwidth. Neurogenesis in HVC thus plays a role in song production and X-ray focal irradiation represents an excellent tool to analyze adult neurogenesis.

KEYWORDS
adult neurogenesis, brain plasticity, photoperiod, Song control system, testosterone

1 | INTRODUCTION

Birds like other living creatures adapt their activity to environmental conditions. Reproduction is one of the most, perhaps the most, costly stage in the annual cycle. It is therefore critical for the individual fitness to select for breeding the most favorable time of the year, when food supply will be abundant to provide the extra energy required by the mother and by the growth of the young. Among environmental conditions, the daily duration of light is the most reliable signal that allows predicting suitable reproductive conditions. Photoperiod changes across the annual cycle are completely reproducible whereas other factors such as temperature or food availability show important

random fluctuations from year to year around an underlying average cycle. Many avian species thus use the annual changes in light: dark cycle to determine when to initiate their reproductive effort.[1–3]

Schematically, four great types of responses to photoperiod could be distinguished. Since food will in general be available in abundance during the summer, some species will start breeding when the photoperiod reaches a critical amount of daily light that is often around 12 (±1) h of light daily (12L:12D; 12 h of light and 12 h of darkness). They remain sexually active as long as the photoperiod is longer than this critical 12 h-duration and their gonads only regress in the fall (Figure 1A). The wood pigeon (*Columba palumbus*) is an example of this strategy that is only possible because they feed on a variety of vegetables (tree buds, weed leaves, seeds, and fruits) that are available for an extended period.[4]

Many species have, however, a much shorter breeding season. They start breeding when photoperiod exceeds the critical value (around 12 h) but the gonads will regress after a period of a few weeks (ranging from 4 to 10 in many species) well before the summer solstice (i.e., while photoperiod is still increasing). It is said that they become photorefractory (Figure 1B). In evolutionary terms, it is considered that this reproductive strategy developed and was selected to avoid that birds start producing nestlings that would not have enough time to grow and eventually migrate before the conditions deteriorate in the fall. It should be noted that, as an additional refinement in adaptation, the critical period that stimulates the initiation of breeding and the duration of exposure to long days before they become photorefractory are adapted to the latitude where the species lives. Living at higher latitude is associated with a longer critical photoperiod for initiating breeding and a shorter duration of exposure to long days before becoming photorefractory. Murton and Westwood (1977) for example compiled for 17 species of captive geese from the *Anser* and *Brenta* genus living at the same location in the UK (Slimbridge or Peakirk wildlife reserves) the median date of first egg laid as a function of the mean latitude where the species lives in the wild.[4] These two variables were linearly and positively correlated ($r = 0.93$ and $r = 0.86$) with the first egg being laid 20 days earlier for species living 25°–30° more south. In addition, the rate of photorefractoriness development increases with longer day lengths[5] so that birds living at high latitudes become photorefractory faster than birds living in the temperate zone. This is adaptive since conditions for breeding remain suitable for breeding during only a very short period in the Arctic.

Japanese quail (*Coturnix japonica*), a species whose reproduction has been extensively studied, continuously breeds in captivity when exposed to long photoperiods (e.g., 16L:8D).[6] Therefore it was initially thought that this species was responding to photoperiod like wood pigeons do (i.e., remain active as long as photoperiod is above the critical value; model illustrated in Figure 1A). However, a detailed study performed on captive quail maintained under a natural photoperiod in Bristol (UK) demonstrated that their gonads start regressing soon after the summer solstice when the photoperiod begins to decrease but is still significantly longer than the photoperiod that initiated breeding in the spring (Figure 1C).[7] This type of response to photoperiodic changes has been called "relative refractoriness" as opposed to the "absolute refractoriness." In relative refractoriness, gonads can still regrow if photoperiod increases again whereas in absolute refractoriness gonadal recrudescence will not happen again until birds have been exposed to short days (to "break refractoriness").[7] Relative refractoriness is explained by a shift in the minimal critical

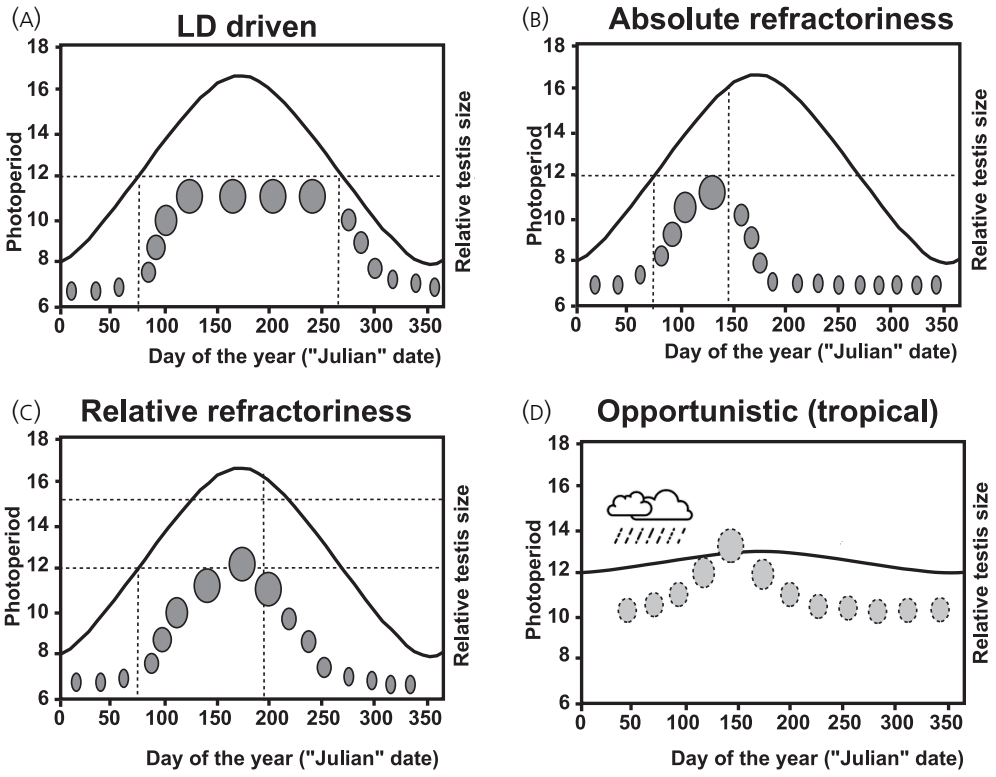

FIGURE 1 Representation of the different relationships that have been observed between the phenology of reproduction (schematically illustrated here by the relative testis size) and the annual changes in photoperiod. Panels (A–C) concern species living in the temperate (or arctic) zone where large changes in photoperiod are observed across the year. They schematically illustrate species that reproduce continuously as long as days are long (A) or that show absolute (B) or relative (C) refractoriness. Panel (D) essentially refers to tropical species where the minor changes in photoperiod only have limited impact on the phenology of reproduction that is largely controlled by supplementary factors such as rain and the associated increased availability of food.

photoperiod needed to sustain reproduction following exposure to long days. For example, if quail are maintained under a long photoperiod of 20L:4D, there is a shift in the critical day length from 12 to 15 h of light; a 14L:10D will thus be considered a short day while it was a long day for birds previously held under 8L:16D.[7]

It should finally be noted that these models of photoperiodic responses concern species living in the temperate or Arctic zone where large changes in day length occur across the year. These species were initially the most studied, but investigations of tropical species increasingly carried out during the last few decades demonstrated that in this environment reproduction is poorly, if at all, controlled by the photoperiod that only shows minor variations across the year.[8] Other factors such as rainfall and the resulting changes in food availability play a more important role.[9,10] Since these factors vary less systematically, reproduction is not tightly linked to the calendar date. These birds often are opportunistic breeders: they maintain across the year a moderate development of the gonads so that they are ready to breed whenever conditions become suitable (Figure 1D). Some temperate zone species also breed in an opportunistic manner as tropical species do. These species can breed at irregular intervals as a function of food availability. Crossbills (*Loxia curvirostra*), for example, feed on conifer cones and can breed at any time between January and August as long as cones are available. No breeding occurs, however, in the fall when birds are molting.[11]

This brief overview is only meant to introduce in a schematic manner the experimental studies described below. More detailed reviews on the topic, older and more recent, and on the underlying endocrine mechanisms are available.[4,12–16]

2 | PHOTOPERIODISM IN CANARIES

Wild canaries (*Serinus canaria*) originate from the Macaronesia archipelagos, a collection of small islands off the west coast of Africa, including five mains clusters: Azores, Madeira, Selvagens, Canary Islands, and Cabo Verde.[17] Canaries are found in only three of these groups of islands: Azores, Madeira (that belong to Portugal), and Canary Islands (that belong to Spain).[18] These islands are located in the low temperate zone between 27° and 39° north and are therefore experiencing significant annual changes in photoperiod (from about 10 to 14 h of light daily). One set of studies analyzed the breeding biology and singing behavior of wild canaries in Ilheu Chao, an uninhabited island of Madeira. It was found that breeding takes place from January–February to July, presumably triggered by the increasing photoperiod.[19,20] Song quality also changes across the year[21] (reviewed in the study by Leitner[18]).

The first canaries arrived in Europe in the 15th century and quickly became a popular pet.[22] They were bred in large numbers and simultaneously selected for different features. About 30 breeds or strains are currently recognized that have been selected for their posture (e.g., Border, Fife Fancy, Gloster, Yorkshire, etc.), their song (e.g., Malinois Waterschlager, Harzer Roller, American singer, etc.), or their color (breeds defined by their color: yellow, white, brown, isabelle, etc.). This selection for arbitrary traits has also unknowingly affected other behavioral or physiological variables.[23,24] Selection for an abundant and complex singing activity seems, for example, to have extended the breeding period and thus affected its control by environmental factors. Also, serum testosterone concentrations are lower in the American Singer than in the Border canaries and testosterone has more extensive effects on brain plasticity in the latter strain.[25]

The Border canary has a reproductive physiology that is supposedly close to the native wild species. Multiple studies carried out in the 1970s in England indicated that when exposed to LD, these birds develop absolute refractoriness within a few weeks.[26–29] In contrast, canaries of the American Singer breed do not develop photorefractoriness as defined by a regression of testes size after chronic photostimulation.[30] The photoperiodic responses of canaries thus markedly differ between canary strains.

3 | THE FIFE FANCY CANARY BREED

We initiated in the 1990s a research program on the relationships between brain plasticity and song control in male canaries. For purely pragmatic reasons (local availability of birds), these experiments were largely carried out with the Fife Fancy breed. This breed was developed relatively recently (1950s) around the county of Fife in Scotland in an attempt to decrease the size of the Border canary back to the wild type size and it quickly became very popular. It is generally considered as a diminutive version of the Border and is thus expected to show photoperiod responses relatively similar to responses of Border canaries and thus to the wild type. Our initial studies were simply carried out on birds in breeding condition but we more recently decided to investigate the photoperiodic control of reproduction in these birds in order to get a better control over experimental designs.

4 | SEASONAL CHANGES IN FANCY FIFE CANARIES

In the first set of studies, we followed the reproductive physiology and singing behavior of a group of 4 Fife Fancy male canaries during a period of 2.5 years. Birds were held in an inside aviary (20–22°C throughout) exposed to a photoperiod that was adjusted each month to match the external photoperiod at 52° north, the latitude of Belgium (range 8L:16D in December to 16.5L:7.5D in June).[31] Birds were over 2 years old when they arrived in the laboratory and they were kept together in one cage (90 cm × 40 cm × 35 cm) throughout. Their songs were recorded during each season. Birds were moved to individual sound-attenuated boxes, allowed to habituate for 3 days, and then recorded for two successive days during the first 2 h after lights on. On this occasion, a small blood sample was collected from the wing vein and assayed for testosterone concentration. Song recordings were analyzed with the Raven Pro v1.4 software (Version 1.4, The Cornell Lab of Ornithology, Ithaca, NY).

This experiment demonstrated that plasma testosterone concentrations, reflecting the activity of the hypothalamo-hypophyso-gonadal axis, changed significantly over time, started

to increase in the spring and peaked each year during the summer (Figure 2A).

Various features of songs varied in parallel (see the study by Cornez et al.[31] for detail). In particular, song duration and amplitude repeatedly increased in the spring and summer and then decreased reaching an annual low in the fall (Figure 2B,C). In the fall of each year, the song of these males also became more plastic as reflected in the increased values of their entropy (Figure 2D).

These data confirmed that male Fife Fancy canaries are still very sensitive to changes in the annual photoperiod that are able, in the absence of changes in temperature or food, to modulate the reproductive activity. The study did not, however, allow to decide whether these canaries develop refractoriness when exposed to LD; their testicular activity regressed in the fall but the resolution in time of the study did not permit to decide whether gonadal regression was taking place before or after the decrease in photoperiod (absolute versus relative refractoriness; see the study by Robinson and Follett[7]).

5 | SINGING BEHAVIOR IN PHOTOSENSITIVE MALES TRANSFERRED TO LONG DAYS FOLLOWING EXTENDED EXPOSURE TO SHORT DAYS

A careful analysis of data from these annual cycles suggested that the recovery of singing behavior (illustrated here by song duration and amplitude) was beginning in the spring and sometimes reaching its maximum while testosterone concentrations were only beginning to increase. This corresponds to what has been described in several species living in the wild. For example, in blue tits (*Cyanistes caeruleus*) living in Corsica the testes volume, singing activity, and the volume of song control nuclei in the brain already reach near maximal values in February when plasma testosterone concentrations are still very low and have barely increased from the winter nadir.[32,33] A similar asynchrony (largely developed song control nuclei when circulating testosterone is still low) has also been observed in song sparrows, *Melospiza melodia morphna*.[34] This partial reactivation of the reproductive activity when photoperiod is still relatively short (below what is usually considered as the critical day length) is supposed to be associated with the end of photorefractoriness triggered by short days.[13,35,36]

Data presented above[31] and other studies from our laboratory were similarly suggesting an early recrudescence of part of the reproductive physiology in the absence of high testosterone concentrations. This was confirmed in a following experiment that was carried out with a group of Fife Fancy males that had been kept, for reasons beyond our control (research interruption due to COVID-19) during an extended period under a short day photoperiod (8L:16D). It was noted during random spot checks that these birds had developed a high singing rate. A study was therefore initiated with two goals in mind: (1) to determine whether exposure to LD would further increase singing activity and (2) to test whether these males would develop photorefractoriness after extended exposure to LD.[37]

These males were randomly assigned to two experimental groups: one group ($n = 8$) was switched to LD (16L:8D) for 12 weeks while

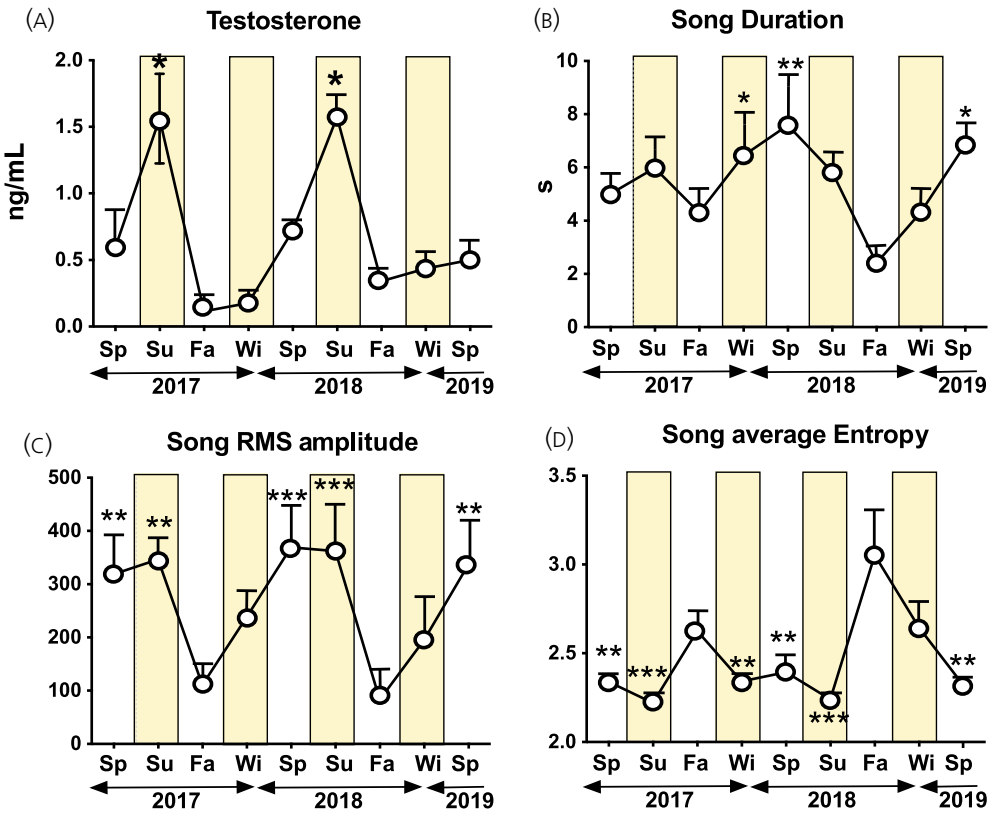

FIGURE 2 Seasonal changes in plasma testosterone concentration (A) and in song duration (B), song amplitude (C), and song entropy (D) in four adult Fife Fancy male canaries exposed indoor during 2 years to changes in photoperiod mimicking the annual light cycle at the latitude of Belgium (52° N). Asterisks indicate the results of post hoc tests (following a significant overall analysis of variance [ANOVA]) comparing the different data points to the 2018 fall. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Fa, Fall; Sp, spring; Su, Summer; Wi, Winter. Error bars are SEM. Redrawn from data in the study by Cornez et al.[31]

the other group ($n = 7$) stayed in the same short photoperiod (8L:16D). Testes size was periodically measured by computer-assisted X-ray tomography (computed tomography scans) with a small irradiator/scanner (Figure 3A). This method was demonstrated to provide nearly identical values as direct measures on the dissected gonads.[38] This is illustrated here by the close correspondence of the images collected by tomography and the view of the gonads after euthanasia: note for example the spheric shape of the right testis and the oblong shape of the left testis visible by both procedures. Birds were kept in individual sound-attenuated chambers and their singing activity was recorded for 2 h starting at 8 a.m. 3 days a week throughout the experiment. These recordings were then automatically analyzed with a custom-made MATLAB script specifically designed to analyze canary song that had been developed by Ed Smith and Bob Dooling (University of Maryland). Its accuracy in measuring various features of male canary song had been confirmed in a previous study.[39]

Quite interestingly, the size of testes that was already large in the SD condition (similar to what had been observed in reproductive birds during previous experiments; see the study by Chiver et al.[37]) did not increase further after transfer to LD; it actually slightly decreased though not significantly (Figure 3B). Circulating testosterone concentrations increased in LD (Figure 3C) but the increase was associated on week 11 with a very large individual variation: the peak is essentially reflecting one bird with a very high concentration. This had apparently no impact on the size of the cloacal protuberance, a well-known androgen-dependent structure that increased progressively in the same manner in both groups of birds (Figure 3D).

Effects of the transfer to LD on singing activity were similarly modest. There was a slight decrease in the average song rate but this change did not reach statistical significance (Figure 3E). The same conclusion was reached for most other song features (small nonsignificant decreases or increases) including the percentage of time spent singing, the average song duration, the song power, entropy, and bandwidth. One feature of song was, however, markedly affected: it is the number of long trills included in these songs. Long trills were defined here as fast repetitions of the same syllable and long trills as more than 15 repetitions of the same syllable, all trills with less than 3 milliseconds between syllables. These long trills thus presumably represent, in

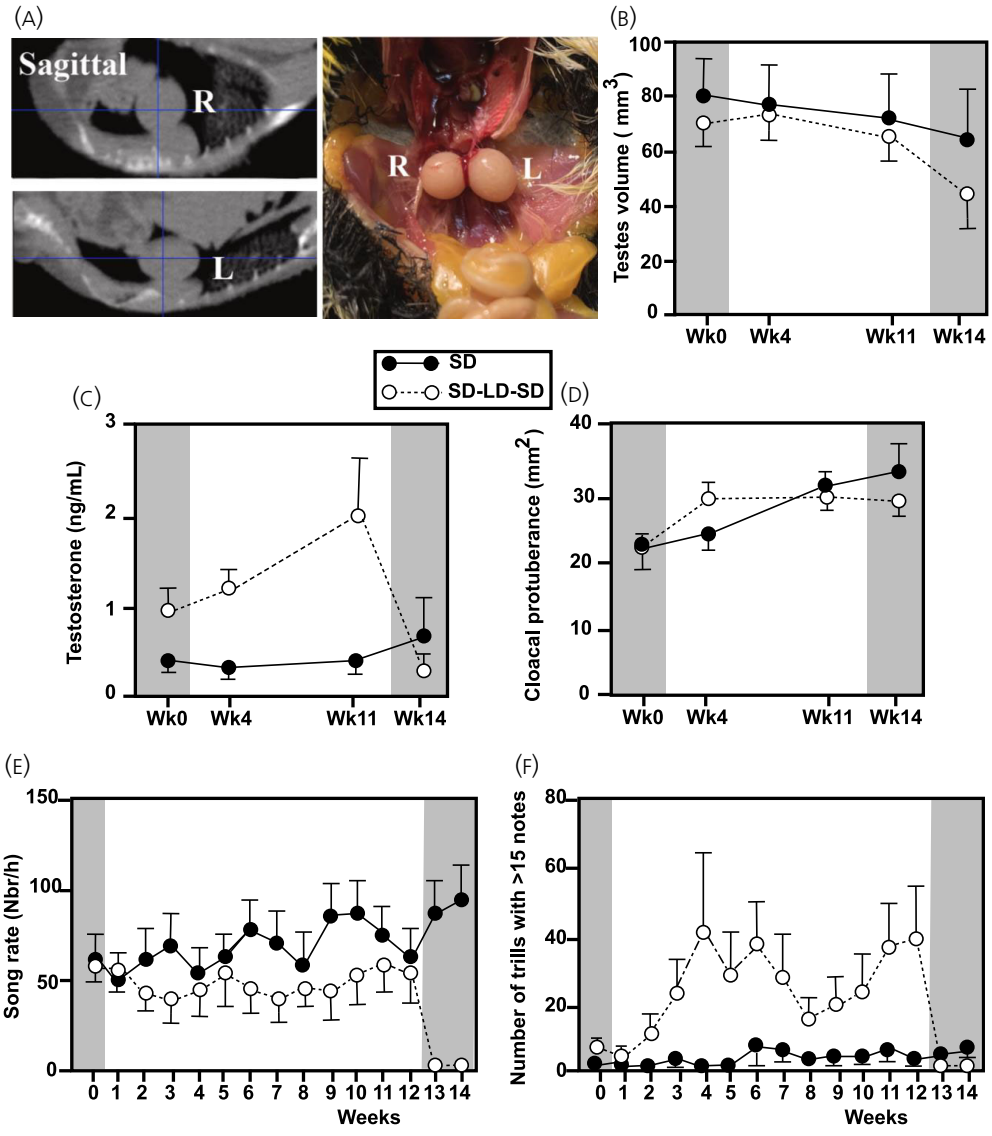

FIGURE 3 Changes in testes size (A, B), testosterone concentrations (C), testosterone-dependent cloacal protuberance (D) and two song features—song rate (E) and rate of long trills (F)—in male Fife Fancy canaries who had been exposed for an extensive period to short days (SD; 8L:6D) and then were transitorily moved to long days (SD-LD-SD; 16L:8D) before being returned to short days again. Short day periods that are indicated by Gray area indicate periods when the LD birds were exposed to SD. See text for additional explanations. Data are means + SEM. Redrawn from data in the studies by Chiver et al.[37] and Lallemand et al.[38]

part or in their entirety, sexy syllables as previously defined and found particularly attractive by females.[40–42] Trills were essentially not present in the songs produced by males when in SD but, in contrast, their occurrence frequency, especially the frequency of trills including more than 15 repetitions, was quite high after transfer to LD (Figure 3F).

In the birds transferred to LD, there was no sign of regression of the testes size and of the testosterone-dependent variables (cloacal protuberance, song rate, and song features) even after 12 weeks. This suggested that these birds do not develop absolute refractoriness like Border canaries do. Indeed in the experiment of Hurley and collaborators that was used to model the current experiment, gonads of males and females had begun regressing after 10 weeks in only 14L:10D and plasma testosterone was back to baseline.[43] Note, however, that in an earlier experiment, the onset of photorefractoriness, defined by a decrease to half maximal size of the left testis, was shown to occur only after 134 days in 16L:8D and 104 days in 20L:4D.[27] A longer exposure to LD would thus be needed to confirm that absolute refractoriness does not develop in Fife Fancy canaries but the fact that even a most sensitive song feature such as trills frequency showed no sign of decrease after 12 weeks strongly suggests that this is the case.

After the 12 weeks in LD, birds were transferred back to 8L:16D and this very rapidly (within a week) induced a crash in the testosterone-sensitive song features (see song and trill rates in Figure 3E,F), presumably explained by the decrease in testes size and plasma testosterone concentration that was documented 2 weeks later (Figure 3B,C). After this return to SD, most song features were actually lower than in birds that had been maintained in SD throughout.

In conclusion, the lack of regression under LD suggests that Fife Fancy canaries do not develop absolute refractoriness even if a longer duration of exposure to LD should possibly have been tested. If we assume that the SD birds represent the photosensitive nonphotostimulated condition, one must then suggest that birds that went to LD for 12 weeks had developed a form of relative photorefractoriness. One cannot exclude however that the birds' physiology was simply following changes in photoperiod duration (option (A) as opposed to (C) in Figure 1). Demonstration of relative photorefractoriness would require that the bird's physiology regresses under a day length that is still longer than the critical photoperiod that initially induces gonadal development and this was not directly tested here.[7] The Fife Fancy canaries thus probably do not react to changes in photoperiod as Border do, they only develop relative refractoriness or they simply follow changes in photoperiods as pigeons do. They are thus less sensitive to photoperiodic changes than the Borders, at least those that were used in the previous experiment of Hurley and collaborators.[43]

6 | SINGING ACTIVITY IN CASTRATED BIRDS HELD UNDER SHORT DAYS

The high song rate observed in males maintained in SD was, as already mentioned, reminiscent of the early recovery of singing activity observed in early spring in several species of birds.[32–34] In fact we had already observed a similar phenomenon during another experiment on another group of male Fancy Fife canaries.[39] These birds had been castrated in preparation for an experiment investigating the activation of song by steroids but, for reasons beyond our control (sound-attenuated boxes used for another experiment), they sat in SD (8L:16D) for another 5 months. We discovered at that point that most of these males were singing actively. At the individual level, this singing activity was not correlated with the circulating concentration of testosterone as measured by enzyme immunoassays. Furthermore, a combined treatment of half of these males with the aromatase inhibitor ATD and the antiandrogen flutamide failed to markedly decrease the singing behavior (see the study by Shevchouk et al.[39] for the minor alterations that were observed). Assays performed by the more specific gas chromatography/mass spectrometry technique at the end of the experiment showed that these treatments had not affected the concentration of testosterone or estradiol in the serum, concentrations that were really basal and close to zero.

Taken together, these data suggest that after a long deprivation of sex steroids, as observed in birds kept in SD and even more in the present birds that had been castrated, a steroid-dependent behavior such as singing can be activated in the presence of extremely low levels of androgens. The behavior has thus either become supersensitive to androgen action or is activated by other neurochemical mechanisms that do not rely on androgen action (see the study by Shevchouk et al.[39] for additional discussion).

7 | SEASONAL AND TESTOSTERONE-INDUCED CHANGES IN SONG CONTROL

Singing in oscines is controlled by a specialized network of brain nuclei. This network includes a caudal circuit mediating song production (HVC [proper name] projecting to the robust nucleus of the arcopallium, RA, and then to the motoneurons innervating the syrinx) and a more rostral circuit implicated in song learning (HVC projecting to Area X of the medial striatum, then to the medial part of the dorsolateral thalamic nucleus, DLM, the lateral magnocellular nucleus of the anterior nidopallium, LMAN and then back to RA).[44,45] The volume of these nuclei changes annually[46] and the seasonal variations in blood testosterone are a main driver of these changes.[47–50] The annual changes in HVC volume were shown to reflect in part changes in the rate of neurogenesis and many studies were devoted to the analysis of this process.[51,52] We report in the second part of this review a selection of these studies that were performed in our laboratory.

Song control nuclei volumes were measured in all Fife Fancy males at the end of the experiment described in the last section with two questions in mind: (1) how large were these volumes in SD males singing actively compared to the maximal volumes observed at the peak of the reproductive season or after treatment with exogenous testosterone, and (2) had these volumes been affected by the temporary exposure to LD and if so how?

HVC and RA volumes in the SD birds were actually quite large and very close to the maximal values observed before in the spring or

after testosterone treatment (see the two left-most columns compared to the others in Figure 4). The maximal values presented in the figure are also very similar to values that have been measured in several experiments from our laboratory investigating for example the development of these nuclei during ontogeny from hatching to adulthood,[53] the effects of social conditions on singing activity and song control nuclei in testosterone-treated birds[54] or simply the effects of testosterone on these dependent variable in castrated males.[55] In agreement with the intense singing activity, the song control nuclei thus develop in these birds under SD to nearly maximal levels as had been observed in blue tits studied in the wild in Corsica.[32]

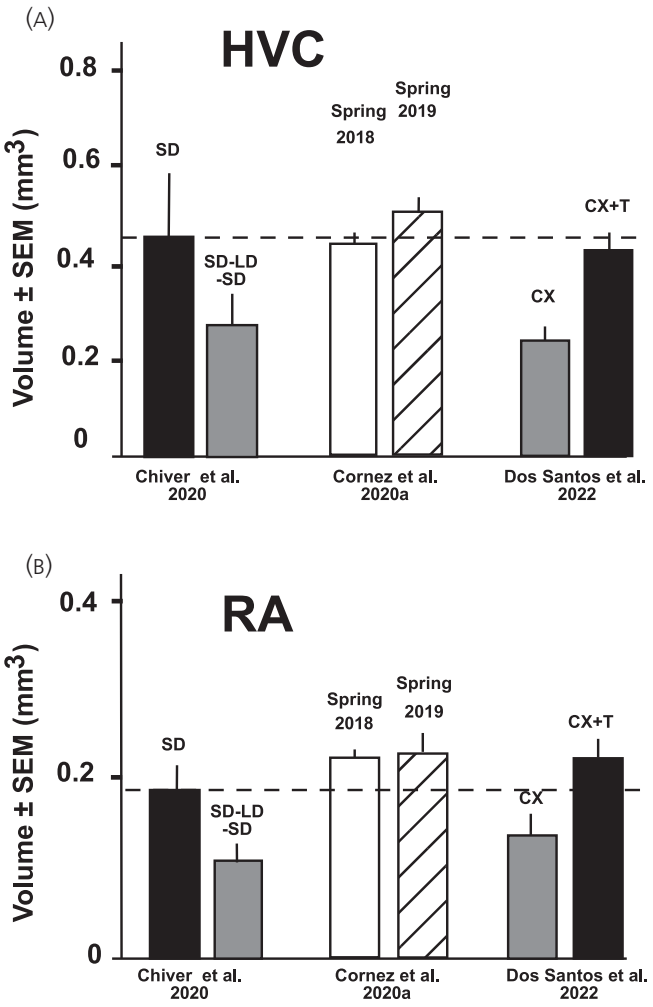

FIGURE 4 Volumes of the song control nuclei HVC (A) and RA (B) measured in the Fife Fancy males that had been kept for an extensive period in short days (SD) or after they had been moved to long days for 12 weeks but then returned to SD 2 weeks earlier (SD-LD-SD). Data are also compared with volumes that were measured in two sets of males at the peak of the reproductive season in spring 2018 and 2019 and in castrated birds that had been treated with exogenous testosterone (CX + T) or with control empty implants (CX). Redrawn from data in the studies by Cornez et al.,[31] Chiver et al.,[37] and Dos Santos et al.[56]

Interestingly, the return to SD after 12 weeks of LD exposure resulted in a major decrease of HVC and RA volumes. These volumes in the SD-LD-SD males were actually close to the volumes previously observed in castrated males (see data from the study by Dos Santos et al.[56] in Figure 4) (see also similar data in the study by Cornez et al.[55]). These volumes thus presumably correspond to the (nearly) maximal regression that can be observed during an annual cycle. Photoperiodic manipulations thus affect brain structure as well as behavior in Fife Fancy canaries.

8 | CHANGES IN HVC VOLUMES REFLECT CHANGES IN NEUROGENESIS

Soon after they discovered that, in canaries, HVC volume varies as a function of seasons[46] and after treatment with exogenous testosterone,[47] Nottebohm and colleagues collected the first evidence indicating that, contrary to the commonly held belief at the time, new neurons are added to this nucleus throughout the life of the subjects.[57] The scientific community was initially unconvinced but evidence accumulated, ultimately leading to the broad acceptance of the idea.[58,59]

Multiple studies then demonstrated that changes in HVC volume are correlated with changes in the rate of neurogenesis, more specifically with the rate of incorporation and survival of the new neurons in the nucleus[60–62] (reviewed in the studies by Nottebohm[51] and Brenowitz[52]). All these initial studies had been carried out by injecting an exogenous marker (tritiated thymidine or bromodeoxyuridine [BrdU]) that is incorporated into DNA during the replication that precedes meiosis. These markers then remain detectable in the daughter cells as long as they do not divide themselves multiple times which would dilute the label. This procedure specifically labels dividing cells only during the limited period during which the markers remain available in the blood for incorporation, a period that was estimated to be limited to a maximum of 2 h[63] (see the studies by Balthazart and Ball[50] and Balthazart and Ball[64] for review). This and other limitations led to the investigation of other possible markers of new neurons.[64] Young neurons are expressing during a limited period of time a number of molecules such as nestin, Ki67, PCNA, or doublecortin (DCX) that can potentially be used to identify them.[64]

During the last 15 years, we established the use of DCX as a marker of neurogenesis in HVC[65,66] and used this marker in a series of studies investigating physiological and behavioral controls of this process. This allowed us to obtain a quantitative measure of neurogenesis across a variety of conditions. We namely demonstrated that the production of new neurons is increased in photostimulated as compared with photorefractory males,[66] in testosterone-treated castrated males as compared with castrates,[56,66,67] and in castrated testosterone-treated males housed with a female as compared with a male.[66] One study also focused on the density of DCX-positive cells at the level of the ventricle wall and showed that this density is increased by a treatment with exogenous testosterone, suggesting a role of the steroid on the rate of multiplication of neuroblasts.[68]

We wondered whether the males held in SD for an extended period would similarly show a high rate of neurogenesis as quantified by the number of DCX-positive cells. As could be expected based on the large volume of HVC in these birds (see Figure 4 in previous section), large numbers of DCX-positive neurons were identified in the SD birds of the experiment and these numbers were drastically reduced in the males that had been exposed to LD for 12 weeks before being returned to SD.[37] These data therefore further support the notion that HVC neurogenesis correlates with the development of this song control nucleus and the concomitant expression of active singing behavior.

9 | FOCAL X-RAY IRRADIATION AND THE FUNCTION OF NEW NEURONS IN ADULT CANARIES

A large number of studies have now identified correlations between singing activity, volumes of song control nuclei, and neurogenesis in HVC. Initial work suggested that larger HVC volumes relate to increased song repertoire.[46] This view was later challenged by negative results[69–71] and it was proposed that HVC volume (and possibly rate of neurogenesis) rather relates to the amount of singing [72] but also possibly to song quality,[73] in particular song stability.[74] However, work testing in a causal manner the function of new HVC neurons has been scarce. The most notable study addressing this question used targeted photolysis of HVC neurons that project to RA. RA was stereotaxically injected with chlorin e6-conjugated nanospheres. After retrograde transport of these spheres, HVC was stereotaxically illuminated by a 674 nm laser light beam that activated the release from chlorin e6 of cytotoxic singlet oxygen resulting in the apoptosis of the neurons that had been retrogradely labeled.[75]

As anticipated, the procedure killed a large number of RA-projecting neurons and this induced an increased recruitment of new neurons. The same procedure applied to HVC neurons projecting to Area X (via injection of the retrograde tracer in X) similarly induced a large and specific neuronal death in HVC but this was not associated with an increased recruitment of Area X projecting neurons. This is in agreement with the well-established fact that Area X-projecting neurons are all born during ontogeny (essentially in ovo) and not replaced in adulthood contrary to the RA-projecting neurons.[76,77]

Behavioral results were more ambiguous. As anticipated, ablation of X-projecting neurons had no short-term effects on song structure since Area X is part of the rostral forebrain pathway implicated in song learning but not production.[45,78–80] In contrast, ablation of RA-projecting neurons induced detectable song deterioration and subsequent recovery after new neurons had been recruited but these effects were observed only in four of the nine treated birds. This individual variation could not be related to any aspect of the experimental procedures (difference in injection site, in incorporation of new neurons, etc.) leaving open a lot of questions in the interpretation of these data.

Analysis of the role of neurogenesis in the control of song is technically difficult. The most common approach to the causal study of neurogenesis in mammals has involved the injection of a variety of antimitotic drugs such as arabinofuranosyl cytidine or methylazoxymethanol acetate that block cell divisions. These compounds have, however, important side effects. They have been characterized in mammals, but avian studies are rare with a few exceptions (e.g., see Hall et al.[81] for a study on hippocampal neurogenesis in black-capped chickadee, *Poecile atricapillus* or Chen and Cheng[82] for a study on hypothalamic neurogenesis in ring doves, *Streptopelia risoria*). They have thus never been used to manipulate neurogenesis in HVC. They would likely diffuse in the lateral ventricles and would probably have major undesired effects obscuring the outcome of the study.

One promising approach was relatively recently developed in studies of mammalian neurogenesis. It is based on the focal irradiation (with gamma or X-rays) of neurogenic zones in the brain.[83–87] Radiation causes DNA breakups and subsequent disruption of protein synthesis and of specific biochemical pathways. Immature rapidly dividing cells are more sensitive than mature neurons to these effects. Therefore neural progenitors are likely to be affected more profoundly than mature neurons. By exposing proliferating precursors to a limited dose of radiation (range 10–20 Gray), it is thus possible to selectively kill them without (too much) collateral damage (see the study by Wojtowicz[87] for a detailed discussion of this specificity aspect). In addition, this irradiation can easily be focalized to the zone of interest, which further increases the specificity of effects. To our knowledge, this approach has never been used in the study of HVC neurogenesis.

We recently carried out one set of experiments testing the feasibility of the irradiation approach in canaries by comparing the same subjects' neurogenesis in the irradiated and control brain hemisphere. A group of adult male Fife Fancy canaries was irradiated via three beams of X-rays (total dose of 23 Grays) focused on the neurogenic zone dorsal to HVC on one brain side while the other side was kept as control (randomly on the left or right side, $n = 4$ in each case). On the day before, birds were also subcutaneously implanted with one 20 mm Silastic™ implant filled with testosterone in order to maximize neurogenesis. Finally, to further assess the rate of neurogenesis, each male received five intra-muscular injections 2 h apart of BrdU at a dose of 50 mg/kg per injection. Birds were kept alive for 2 weeks. They were then anesthetized and perfused with 4% paraformaldehyde. Brains were dissected out of the skull, postfixed for 24 h in paraformaldehyde, cryo-protected in 30% sucrose, frozen on dry ice, and stored at −80°C until used. Brains were cut on a cryostat in 30 µm coronal sections and stained for BrdU and DCX. As done in our previous experiments,[65,66] two types of DCX-positive cells were separately counted: fusiform, uni- or bi-polar, cells that represent very young neurons in the process of migrating to their final destination and multipolar cells that correspond to slightly older neurons that have reached their destination and begun their final differentiation. Numbers of cells expressing these markers were then quantified per unit surface (see the study by Chiver et al.[88] for all technical details).

These quantitative data confirmed that neurogenesis had been markedly inhibited on the irradiated side (Figure 5). BrdU labeling was

observed in DCX$^+$-fusiform neurons and their number was significantly decreased on the irradiated as compared to the control side (Figure 5C). In contrast, no BrdU label was ever detected in slightly older DCX$^+$-multipolar neurons. This was in fact expected because brains were collected only 2 weeks after the BrdU injections and new neurons acquire their final multipolar morphology only after about 1 month at least.[64–66] BrdU labeling was also observed in DCX-negative cells and the number of these cells was also significantly decreased by irradiation (Figure 5C). These BrdU$^+$-DCX-negative cells presumably represent nonneuronal cells born during the BrdU injections, supporting the idea that irradiation depletes progenitor cells irrespective of whether they will later acquire a neuronal or glial phenotype.

Quantification of DCX$^+$ cells, irrespective of whether they are simultaneously labeled by BrdU or not, similarly revealed a depletion of DCX$^+$-fusiform neurons (Figure 5D). Again DCX$^+$-multipolar neurons were not decreased by irradiation. These neurons are indeed older than 2 weeks, the duration between irradiation and brain collection. They were born before the irradiation and this supports the notion that irradiation preferentially depletes progenitor cells without affecting the more mature neurons. Note also that overall DCX$^+$ neurons were present in larger numbers that DCX$^+$-neurons co-labeled by BrdU. This fits in well with the notion that DCX is expressed by new neurons during a long duration (1 month and more) whereas an injection of BrdU labels neurons during only a brief period ranging between one and 2 h.[63] Based on these promising results, a second experiment then investigated the functional consequences of a bilateral irradiation on the development of testosterone-induced singing in female canaries.

This experiment was performed on adult Fife Fancy females that were treated at the beginning with a Silastic™ testosterone implant. It was reasoned that the effects of depletion in neurogenesis would potentially be more prominent on the development of song in a previously inactive female than on established song in males. The neurogenic zone dorsal to HVC was irradiated on both sides of the brain by two horizontal X-ray beams (total dose of 23 grays) originating from both sides of the brain (Figure 6A,B). Seven females were irradiated in this way while seven control females were submitted to control treatments, including anesthesia, in parallel. On the same day, all birds received five BrdU injections 2 h apart (50 mg/kg per injection) to label cells replicating their DNA.

Birds were individually housed in sound-attenuated boxes. Their singing activity was recorded for 2 h starting at lights on (8:00 a.m.) for three successive days each week for 4 weeks after irradiation. The weak singing activity present before irradiation and testosterone implantation was also recorded as a baseline. Song recordings were quantitatively analyzed in a semiautomatic manner by the MATLAB routine already mentioned before. Brains were collected here after 4 weeks to allow sufficient time for song development in the testosterone-treated females. Brains were processed like in the previous experiment.

As observed previously in males, this irradiation substantially decreased the number of new neurons in HVC (Figure 6). In particular, the density of BrdU-positive DCX$^+$-fusiform neurons was drastically

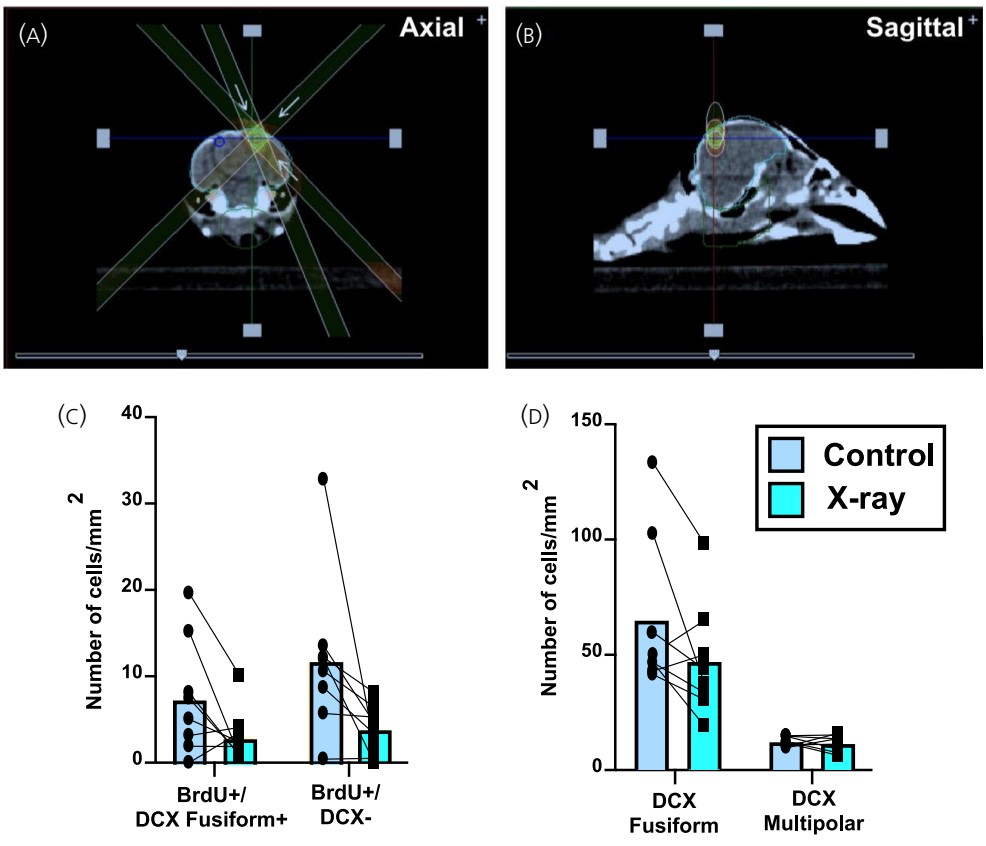

FIGURE 5 (A, B) Copies of the irradiator computer screen illustrating the three X-ray beams that were focalized on the neurogenic role located in the lateral ventricle just dorsal to the right HVC. (C, D) Quantification of HVC neurogenesis in the irradiated and nonirradiated control side as measured by the number of cells that had incorporated BrdU and/or were expressing doublecortin (DCX$^+$). Mean values are presented together with all individual data points connected by a black line. Redrawn from data in the study by Chiver et al.[88]

decreased (Figure 6C). Again, almost no multipolar DCX$^+$ neuron contained BrdU: one single subject in the X-ray group contained one or two BrdU-DCX$^+$-multipolar neurons. Brains were collected here 1 month after BrdU injections, which supports the idea that DCX$^+$ neurons take at least 1 month to acquire a multipolar morphology. BrdU label was also observed in cells not labeled by DCX that presumably are nonneuronal in nature. Once again, the total density of DCX$^+$-neurons was larger than the density of these cells simultaneously labeled by BrdU and X-ray irradiation decreased DCX$^+$ fusiform neurons but not those with a multipolar morphology that were probably born before irradiation. It must be noted that this decrease in neurogenesis was not associated with a decrease in HVC volume. This is probably explained by a number of reasons including (a) the fact that HVC volume is not controlled only by the rate of neurogenesis but also by changes in cell spacing, neuronal dendritic arborization, number of glial cells, and so forth; (b) the decreased neurogenesis only lasted for 4 weeks which was not sufficient to affect the number of mature neurons (multipolar DCX-ir cells) but only the younger ones (fusiform DCX-ir) that occupy less volume; and (c) the decrease in neurogenesis was only partial (about 50%) and larger depletions, as could possibly be obtained by repeated irradiations or by irradiation at a higher dose, might possibly be needed to affect the overall HVC volume.

However, the decreased neurogenesis was here bilateral and could thus potentially affect singing behavior. Female song output was extremely low before irradiation and implantation with testosterone Silastic™ capsules: three females only produced less than one song per hour (Figure 6E). Testosterone increased the singing motivation and fine-tuned many song features, as previously demonstrated.[56,89] Some of these changes in song rate and structure were affected by the irradiation and subsequent decrease in neurogenesis. Song rate increased more in irradiated females toward the end of the experiment and, in contrast, measures of song entropy and bandwidth were significantly decreased. For these three variables, a significant interaction between treatment and time was observed in the two-way ANOVA of these data. Other features that were quantified were not significantly affected (see the study by Chiver et al.[88] for details). Note that the negative values of the logarithm of the Wiener entropy presented here were calculated on the entire songs (not syllables) and can thus be influenced by many aspects of the song structure. More

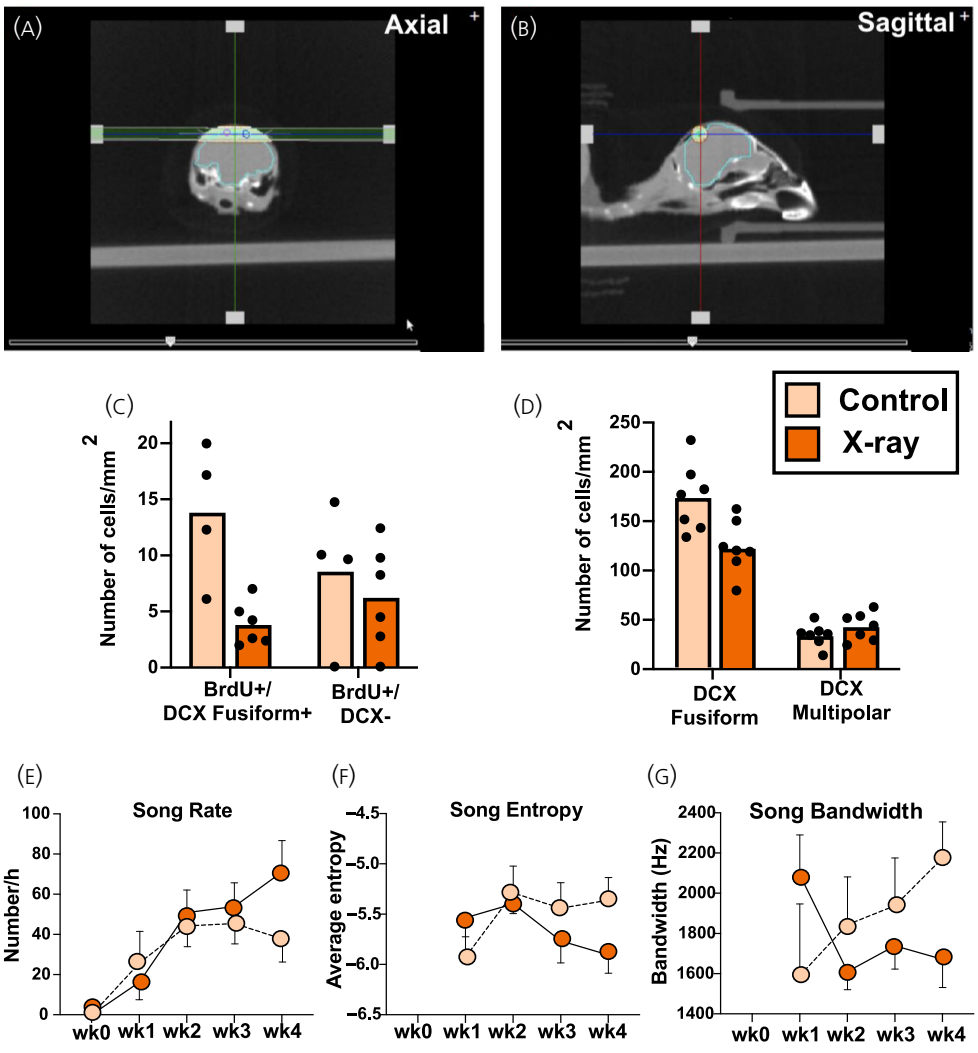

FIGURE 6 (A, B) Copies of the irradiator computer screen illustrating the two X-ray beams that were focalized on the neurogenic zone located on both sides of the brain in the lateral ventricle just dorsal to HVC. (C, D) Quantification of HVC neurogenesis in the irradiated and control nonirradiated birds as measured by the number of cells that had incorporated BrdU and/or were expressing doublecortin (DCX). Mean values are presented together with all individual data points. (E, F) Song rate and song features observed in the testosterone-treated female canaries in which HVC had or had not been irradiated to deplete new neurons incorporation. Data in (E–G) are means + SEM. Redrawn from data in the study by Chiver et al.[88]

negative values represent songs that are less structured and less stereotyped: for example, we demonstrated that the songs of castrated males are characterized by more negative values of entropy than the songs of testosterone-treated birds. The more negative values might reflect, among other things, the decreased bandwidth but other features such as the slight, nonsignificant, decrease in the number of syllables per song or any other feature not quantified by the Matlab routine could also contribute and should be investigated. This less stereotyped song is reminiscent of the effects observed after inhibition of androgen action in HVC. Androgens increase the incorporation of new neurons in HVC[90] and implantation of an androgen receptor blocker in HVC increases overall song variability by increasing the bandwidth coefficient of variation and the variability in syllable-type usage and syllable sequences.[91]

Note also that these changes in entropy and bandwidth were significantly correlated at the individual level with the level of neurogenesis depletion, especially as measured by the decrease in the density of DCX^+-fusiform BrdU-positive neurons (see the study by Chiver et al.[88] for details of these analyses). This supports the conclusion that the changes in song structure are really caused by the inhibition of neurogenesis rather than by a nonspecific effect of the irradiation, as would have been observed if a global lesion of HVC had been produced by the irradiation.

The increased song rate in irradiated birds might seem a priori counter-intuitive but it might be reminiscent of the very high song rate observed during the period of plastic song in ontogeny[53] and during the plastic phase of song production in the fall and winter in adult birds of this species.[31] Since birds at that time are producing mostly imprecise and variable songs, it might be speculated that they are practicing more frequently in an attempt to correct by trial and errors.

10 | CONCLUSIONS

This suite of experiments demonstrates that Fife Fancy canaries have retained a delicate sensitivity to changes in the daily photoperiod, fairly similar to the reactions of Border canaries even if they do not seem to develop absolute refractoriness. They display annual cycles in testosterone concentration and singing behavior in response to cyclic changes in the daily numbers of hours of light. Their activity is however not only a response to the photoperiod: testes, song, and song control nuclei develop after exposure to short days when birds and supposedly photosensitive, and these features completely regress in photostimulated birds returns in SD. The rapidity and magnitude of this regress suggests that birds have become photorefractory even if additional work would be needed to precisely identify the underlying mechanisms.

HVC development essentially correlates with changes in singing behavior and partly relies on changes in local neurogenesis. The specific role of the new neurons has been poorly investigated in a causal manner but we identified and validated a new procedure based on local irradiation to selectively deplete neurogenesis and assess functional impacts on singing behavior. All these data confirm that canaries continue to represent a very useful model to investigate the mechanistic bases of brain plasticity mediating singing behavior.

AUTHOR CONTRIBUTIONS

Jacques Balthazart: Conceptualization; data curation; formal analysis; funding acquisition; project administration; visualization; writing – original draft.

ACKNOWLEDGMENTS

Research described in this review was supported in part by a grant from the National Institute of Neurological Disorders and Stroke (Grant RO1NS104008). The experimental work summarized here was carried out over the years with the help of multiple PhD students and post docs that cannot be all cited but include as major contributors: Gilles Cornez, Olesya Shevchouk and Géradine Boseret (PhD students), Jennifer Barker, Ednei Barros dos Santos, Ioana Chiver, and Shelley Valle (post docs). Song analysis was carried out in part with a MATLAB routine generously provided by Ed Smith and Robert Dooling (University of Maryland). This research also benefited from extensive intellectual interactions with my two long-term collaborators Charlotte A. Cornil (University of Liège) and Gregory F. Ball (University of Maryland).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jne.13449.

DATA AVAILABILITY STATEMENT

This is a review paper essentially based on data from my laboratory that have been published in reffereed journals and original data are available.

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How to cite this article: Balthazart J. Photoperiodism, testosterone and adult neurogenesis in canaries (*Serinus canaria*). *J Neuroendocrinol*. 2024;e13449. doi:10.1111/jne.13449