

Early nutritional stress impairs development of a song-control brain region in both male and female juvenile song sparrows (*Melospiza melodia*) at the onset of song learning

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Birdsong is a sexually selected trait and is often viewed as an indicator of male quality. The developmental stress hypothesis proposes a model by which song could be an indicator; the time during early development, when birds learn complex songs and/or local variants of song, is of rapid development and nutritional stress. Birds that cope best with this stress may better learn to produce the most effective songs. The developmental stress hypothesis predicts that early food restriction should impair development of song-control brain regions at the onset of song learning. We examined the effect of food restriction on song-control brain regions in fledgling (both sexes, 23–26 days old) song sparrows (*Melospiza melodia*). Food restriction selectively reduced HVC volume in both sexes. In addition, sex differences were evident in all three song-control regions. This study lends further support to a growing body of literature documenting a variety of behavioural, physiological and neural detriments in several songbird species resulting from early developmental stress.

Keywords: nutritional stress; neural development; HVC; sex differences; birdsong

1. INTRODUCTION

Male song is an important cue for mate choice by females of many songbird species. Females often prefer to mate with males who sing more frequently, with males who sing local song variants and/or with males who sing with greater complexity (Searcy & Yasukawa 1996). One way birdsong could act as an indicator of male quality is when the display carries a cost to ensure signal honesty (Zahavi 1975; Grafen 1990). Within the songbird brain, a network of specialized nuclei, known collectively as the song-control system, controls the learning and production of birdsong (reviewed in Brenowitz *et al.* 1997). Recently, Nowicki *et al.* (1998) proposed that the reliability of birdsong as an indicator of male quality can be maintained by the costly development of song-control nuclei during a period wrought with environmental stressors, not the least being nutritional. During the first or second month of life, most songbirds are both forming auditory memories on which they will base their own song production (sensory phase of song learning) and rapidly developing the brain regions associated with this process. Thus, birds best able to withstand stressors during early ontogeny should develop the most attractive songs (Nowicki *et al.* 1998).

The developmental stress hypothesis predicts that stressors experienced early in life impair brain development, and subsequently, song learning, thereby resulting in less attractive songs. Lab-reared swamp sparrows

(*Melospiza georgiana*), subjected to nutritional stressors early in development, exhibited poorer copying fidelity of model songs as adults when compared with *ad libitum* controls (Nowicki *et al.* 2002b). In addition, the volumes of two song-control regions, HVC and RA (robust nucleus of the arcopallium), were significantly smaller in the food-stressed birds. Nutritional stressors during early ontogeny also contributed to a suppressed humoral immune response and lower overall body weight (Buchanan *et al.* 2003) in juvenile European starlings (*Sturnus vulgaris*). Stressed starlings also sang less frequently, performed shorter song bouts and generally delayed singing behaviour compared with controls, when assessed the following year (Buchanan *et al.* 2003). Spencer *et al.* (2003) found that nutritional stressors significantly reduced zebra finch (*Taeniopygia guttata*) nestling growth rates, and resulted in adult song that was significantly shorter and contained fewer syllables compared with the control condition. Furthermore, HVC volume for the food-stressed finches was selectively reduced compared with the overall brain volume and other nuclei (Buchanan *et al.* 2004).

Recently, support for the developmental stress hypothesis has rapidly grown, showing impacts of developmental stress induced by food restriction or pathogens on both the size of song-control regions in the adult brain and the adult song (e.g. Spencer *et al.* 2004, 2005a,b). Despite this supporting evidence some issues remain unresolved. First, it has yet to be demonstrated that developmental stress impairs neural development of the song-control system prior to the onset of song

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learning. It is possible that developmental stress could reduce song learning and singing by some other means, and therefore, limit development of the brain. Such behaviourally driven limits on brain development have been demonstrated in avian hippocampus development and seasonal plasticity in HVC (Clayton & Krebs 1994; Ball *et al.* 2004; Sartor & Ball 2005). Second, females are subject to stress during development, and early learning impacts their song production and preferences (Riebel *et al.* 2005). There is some evidence that song-control regions, such as HVC, may play a role in song perception in addition to song production (e.g. Brenowitz 1991; Leitner & Catchpole 2002). Several other auditory areas are also specialized for the auditory processing of song (Mello *et al.* 1992), and these may be affected as well by stressors experienced early in life. A second prediction of the developmental stress hypothesis, therefore, is that nutritionally stressed songbirds may be impaired in song perception later in life, in both males and females. Indeed, female canaries (*Serinus canaria*) with smaller HVC sizes are poor at discriminating among conspecific songs (Leitner & Catchpole 2002). Thus, the developmental stress hypothesis may apply to both production and perception of birdsong.

The objectives of our study were twofold: first, to document if the early stressor of food restriction impairs development of the song-control system prior to the onset of song learning and second, to document this phenomenon in both males and females. Our study was conducted on song sparrows (*Melospiza melodia*), which have been extensively studied as a model system in birdsong. Female song sparrows prefer large song repertoires, geographically local and well-learned songs (Searcy 1984; Searcy *et al.* 1997; Nowicki *et al.* 2002a). Indeed, song repertoire size is highly predictive of fitness in this species (Reid *et al.* 2005). In adult male song sparrows, HVC size is correlated with song repertoire size (J. A. Pfaff & S. A. MacDougall-Shackleton 2006, unpublished data). Thus, the song sparrow is an excellent species for testing the predictions of the developmental stress hypothesis. For this purpose, we compared the size of song-control brain regions in juvenile song sparrows (23–26 days old) that were fed *ad libitum* or food restricted as nestlings. At this age, birds are just entering the sensory phase of song learning (Marler & Peters 1987).

2. MATERIAL AND METHODS

(a) *Experimental treatments*

Nestling song sparrows (*M. melodia*) were hand reared in either food-restricted or *ad libitum* fed conditions as part of a study on food stress and stable isotopes (Kempster 2005). Briefly, song sparrow nestlings (both sexes) were removed from their nests on day 3 post-hatch and hand reared in the lab. Nests were randomly assigned to either control or experimental conditions. Controls were fed *ad libitum*, while the experimental group was fed 65% of the mass of the average control diet. In all other respects, the two treatment groups were treated identically. Birds were sacrificed when 23–26 days old using an overdose of isoflurane vapours followed by rapid decapitation. Bodies were collected for isotope analysis, brains were rapidly fresh frozen using

pulverized dry ice, and then stored at -70°C until further processing.

(b) *Tissue processing and measurement*

We analysed the brains of 20 birds (five from each sex and treatment group). Using a cryostat, 40 μm coronal sections were cut and thaw-mounted onto microscope slides. Mounted sections were immediately fixed by immersing in buffered 4% paraformaldehyde (pH 8.5) for 10 min. Next, sections were Nissl stained with thionin, serially dehydrated in ethanol and then protected with cover-slips affixed with Permount (Fisher Scientific).

All measurements were performed by an observer (IFM), blind to sex and treatment of the subjects. Slides were examined with a bright field microscope equipped with a digital microscope camera. Images of sections of the telencephalon and the song-control regions, HVC, area X and RA, were captured. We used imaging software to trace the outlines of these regions and then combined these areas using the formula of a truncated cone to estimate the total volume of each structure. Volume estimates were based on the areas of every 20th section for the telencephalon (800 μm intervals) and every second section for the song-control regions (80 μm intervals).

(c) *Statistical analyses*

We compared male versus female and food-restricted versus *ad libitum* fed birds for each brain region using a series of two-way ANOVAs ($\alpha=0.05$). The sample size was 20 birds (five birds in each of the four treatment groups) except for the following. The borders of area X were more poorly defined in females as compared with males, and we were unable to obtain volume estimates for two *ad libitum* fed females and three food-restricted females and also unable to measure the telencephalon volume for one female. Preliminary paired *t*-tests revealed no significant differences between the left and the right hemispheres for any of the brain areas measured, so all the analyses were performed on summed left and right volumes.

3. RESULTS

Food restriction reduced the size of the song-control nucleus HVC for both males and females (figures 1 and 2; table 1). There was a trend towards a reduction in the overall telencephalon volume (figure 2), but this was not statistically significant. Similarly, there were no effects of food restriction on the volume of area X or RA (figure 2; table 1).

Food restriction led to a decrease in the HVC volume with no interaction with sex (table 1), i.e. food restriction resulted in decreased HVC size in both males and females (figure 2).

Several lines of evidence suggest that the effect of food restriction on HVC volume was not merely a result of overall reduction in telencephalon volume. First, HVC volume was not significantly related to telencephalon volume in this study (linear regression: $r^2=0.018$, $p=0.26$). Second, a two-way ANOVA on the proportion of HVC to telencephalon volume yielded the same results as the analysis of HVC volume (table 1). Thus, food restriction resulted in a specific decrease in the volume of HVC. Finally, we did not detect any significant effect of food restriction on the volumes of area X or RA (table 1),

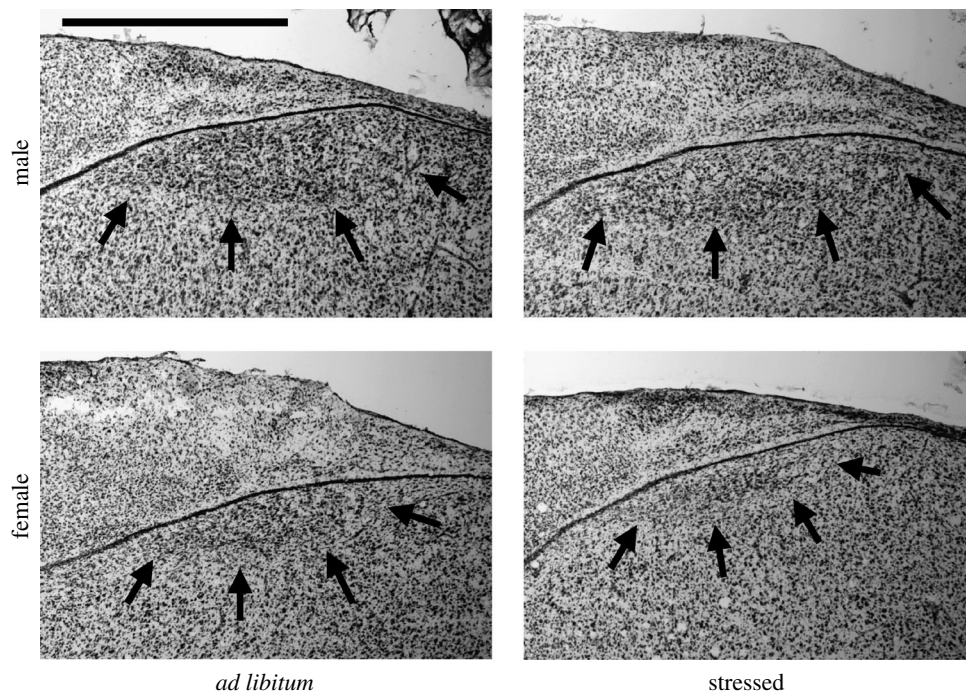


Figure 1. Photomicrographs illustrating effects of food restriction and sex differences on HVC size in juvenile song sparrows. Images are coronal sections of HVC selected from birds typical of each treatment group. The ventral border is indicated by arrows. Scale bar, 1 mm.

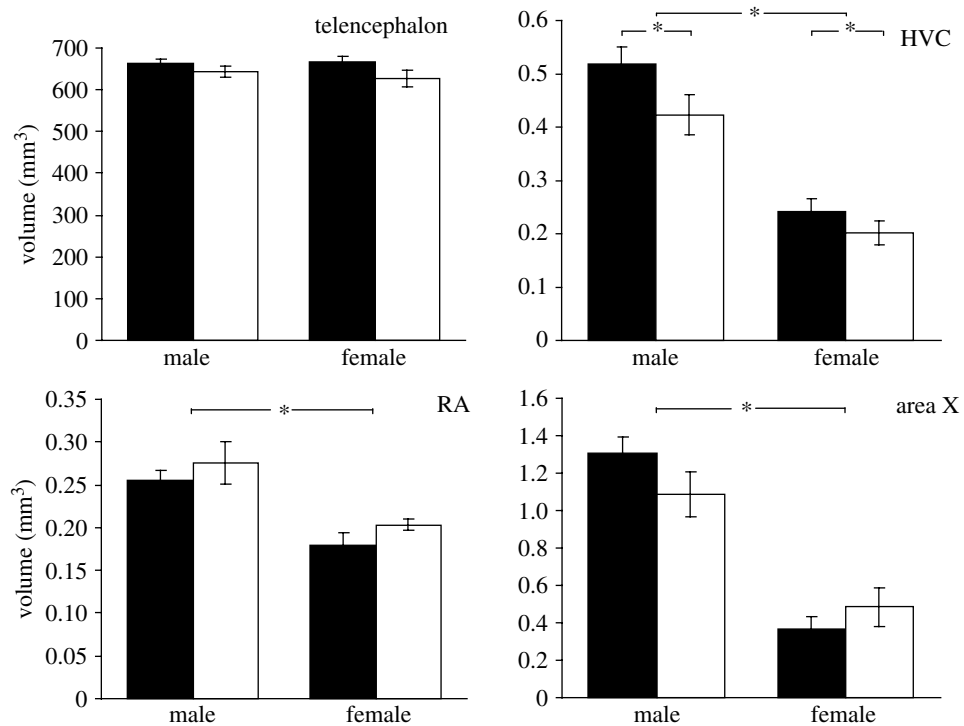


Figure 2. Volumes of telencephalon and three song-control regions in food restricted (white bars) or *ad libitum* fed (black bars) male and female juvenile song sparrows. Bars indicate mean \pm s.e. Asterisks indicate significant main effects determined by two-way ANOVA.

which would be expected if overall reduction in brain size accounted for reduction in the size of the song-control system. All these lines of evidence indicate that HVC is particularly sensitive to the effects of developmental stressors (table 1).

Sex differences were evident in all the three song-control regions measured (figure 2; table 1). The volumes of HVC, area X and RA in females were approximately

47, 72 and 35% that of males, respectively. There was no indication of a sex difference in the overall telencephalon volume (figure 2).

4. DISCUSSION

We demonstrate a selective impairment of HVC development related to early food restriction in song sparrows.

Table 1. Results of two-way ANOVAs comparing volume of telencephalon and three song-control brain regions between food-restricted versus *ad libitum* fed and male versus female song sparrows. (Significant *F*-ratios are indicated in bold.)

brain region	effect of food		effect of sex		interaction	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
telencephalon	<i>F</i> _{1,15} = 2.6	<i>p</i> = 0.13	<i>F</i> _{1,15} = 0.03	<i>p</i> = 0.96	<i>F</i> _{1,15} < 0.01	<i>p</i> = 0.99
HVC	<i>F</i>_{1,16} = 5.4	<i>p</i> = 0.03	<i>F</i>_{1,16} = 72.1	<i>p</i> < 0.001	<i>F</i> _{1,16} = 0.9	<i>p</i> = 0.4
HVC/telencephalon	<i>F</i>_{1,15} = 6.0	<i>p</i> = 0.03	<i>F</i>_{1,15} = 91.1	<i>p</i> < 0.001	<i>F</i> _{1,16} = 0.3	<i>p</i> = 0.6
area X	<i>F</i> _{1,11} = 0.16	<i>p</i> = 0.70	<i>F</i>_{1,11} = 39.1	<i>p</i> < 0.001	<i>F</i> _{1,11} = 1.8	<i>p</i> = 0.2
RA	<i>F</i> _{1,16} = 2.0	<i>p</i> = 0.17	<i>F</i>_{1,16} = 21.7	<i>p</i> < 0.001	<i>F</i> _{1,16} = 0.01	<i>p</i> = 0.9

Our results provide further support for the developmental stress hypothesis as an explanation for how birdsong has been able to evolve as an honest indicator of male quality. Previous studies have measured the effects of early developmental stress on adult phenotype—song and the song-control system (Buchanan *et al.* 2004; Nowicki *et al.* 2002b; Spencer *et al.* 2003, 2004, 2005a,b). Our results show the negative impacts of similar early nutritional stressors on the song system at an extremely young age, coinciding with the time song sparrows begin to acquire song (Marler & Peters 1987) and, presumably, are in the process of rapidly developing HVC. Swamp sparrows (*M. georgiana*), a congener to song sparrows, experience increases in volume and neuron numbers in song-control regions, such as HVC, approximately 20–60 days after hatching (Nordeen *et al.* 1989), and we would expect a similar developmental timeline for song sparrows. The juvenile males in the present study had HVC volumes about half that of the adult breeding males as assessed by similar histological techniques despite having similar telencephalon sizes (J. A. Pfaff & S. A. MacDougall-Shackleton, unpublished data). Thus, HVC was likely still rapidly growing at two weeks of age when we observed the effects of food restriction.

While no behavioural data could be gathered from the present study on song sparrows, swamp sparrows experiencing early nutritional stressors had a decreased ability to copy local song structure (Nowicki *et al.* 2002b), indicating that early food restriction affects adult song production negatively in this genus. In addition, song repertoire size is correlated to the HVC size in adult song sparrows (J. A. Pfaff & S. A. MacDougall-Shackleton, unpublished data). We predict that our manipulation would have resulted in poorer song learning and reduced song repertoire size, but an important follow-up is to assess the accuracy of song learning in nutritionally stressed song sparrows, and to determine the attractiveness of songs from stressed birds compared to *ad libitum* fed birds.

As males perform the majority of singing in many songbird species, much of the research on birdsong has focused solely on male behaviour and anatomy. However, our findings indicate that nutritional stressors affect both male and female HVC development. Another interesting finding is the relatively young age at which these volume differences are present, with the age range being 23–26 days at sacrifice in the present study. Although the sex difference in adult song sparrow HVC size has not been reported earlier, the size of sex difference observed here is similar to that observed in other songbird species with a sex difference in singing behaviour similar to that of

song sparrows (MacDougall-Shackleton & Ball 1999). Male song sparrows need to memorize and later produce song, while females must discriminate between different songs when choosing potential mates. Female preferences for song from the local geographical area have been documented in song sparrows (Nowicki *et al.* 2002a), indicating that quality of song learning could serve as an honest indicator of male song sparrow fitness. Any adverse development within areas of the song system, required for song acquisition, should impair learning in both males and females, perhaps with females showing less discrimination between variable songs once mature.

In support of this idea, female canaries distinguish between ‘sexy’ and ‘unsexy’ songs as measured by copulation solicitation displays. A positive correlation was found between the size of HVC and the difference in the number of copulation displays solicited by sexy and unsexy songs (Leitner & Catchpole 2002). Presumably, females with a reduced HVC from nutritional stress would demonstrate impairment in song discrimination, but unfortunately, this relatively simple hypothesis remains untested. If true, this effect could lead to early stress resulting in both poorer singing and poorer evaluation of song by females, leading to assortative mating between the best song producers and the best song perceivers. Again, this hypothesis has yet to be tested.

While the present study suggests that HVC development is selectively impaired by nutritional stress, the underlying mechanism behind the effect remains elusive. While it is quite possible that reduced energy availability alone restricts the growth and development of HVC, other explanations are being researched. Spencer *et al.* (2003) investigated the role of the stress hormone, corticosterone, as a mechanism through which nutritional stress impacts song system development in zebra finches. Both food restriction and corticosterone treatment resulted in reduced song bout complexity and duration compared with controls (Spencer *et al.* 2003), and also resulted in smaller HVC volume (Buchanan *et al.* 2004). It should be noted, however, that basal corticosterone levels did not differ between food stress and control conditions, questioning the notion that food restriction triggered a general stress response in this study. In addition, glucocorticoids affect energy balance and hence it is possible that corticosterone affected HVC via nutritional or energetic effects rather than the other way around. The authors noted that the failure to detect elevated corticosterone levels in food restricted birds could be a result of sampling on a day when the birds were not experiencing food restriction. Determination of how food restriction and other stressors modify the adrenal stress response and subsequently impair song-system development awaits

further study. Towards this, Kempster (2005) has found that the food-restricted birds in the present study had elevated baseline corticosterone and blood glucose levels. This is consistent with the idea that the adrenal stress response to food restriction negatively influences HVC development, but confirming that requires further experimental studies.

Despite the considerable support generated for the developmental stress hypothesis, there are several areas that require further investigation. As mentioned earlier, the underlying mechanism through which developmental stress exerts its effect on the song system remains uncertain. Similarly, factors contributing to the reduced nuclei volume need explanation. Potential mechanisms included reduced neurogenesis, increased apoptosis or changes in neuron spacing and connectivity with no change in neuron number. How these aspects of neural development are modified by developmental stressors should prove a fruitful area of research.

Another untested prediction of the developmental stress hypothesis is whether or not young males of higher quality (assessed through paternal measurements) are indeed better able to cope with stressors. Buchanan *et al.* (2004) found that the number of syllables in both genetic and foster father song contributed to the variance in offspring HVC size; however, the majority of the studies have not included such rigorous controls. The developmental stress hypothesis highlights the critical role of the development of physiological systems for a complete understanding of the function and evolution of adult behaviour, and should stimulate productive research for some time to come.

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