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Developmental stress selectively affects the song control nucleus HVC in the zebra finch

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Songbirds sing complex songs as a result of evolution through sexual selection. The evolution of such sexually selected traits requires genetic control, as well as selection on their expression. Song is controlled by a discrete neural pathway in the brain, and song complexity has been shown to correlate with the volume of specific song control nuclei. As such, the development of these nuclei, in particular the high vocal centre (HVC), is thought to be the mechanism controlling signal expression indicating male quality. We tested the hypothesis that early developmental stress selectively affects adult HVC size, compared with other brain nuclei. We did this by raising cross-fostered zebra finches (*Taeniopygia guttata*) under stressed and controlled conditions and determining the effect on adult HVC size. Our results confirm the strong influence of environmental conditions, particularly on HVC development, and therefore on the expression of complex songs. The results also show that both environmental and genetic factors affect the development of several brain nuclei, highlighting the developmental plasticity of the songbird brain. In all, these results explain how the complex song repertoires of songbirds can evolve as honest indicators of male quality.

Keywords: song; neural development; stress; *Taeniopygia guttata*; sexual selection; songbird

1. INTRODUCTION

Song complexity is known to be important for mate choice in many songbird species (Andersson 1994; Searcy & Yasukawa 1996), and complex repertoires have evolved by way of female choice on the underlying control mechanisms of song expression in the male (Catchpole 1996). Evolutionary biologists are fascinated by how signal honesty is maintained, and bird song is often a trait of interest. Current models of sexual-selection theory suggest that for sexual traits to evolve as honest indicators of male quality, they should be costly to produce or to maintain (Zahavi 1975; Pomiankowski *et al.* 1991; Andersson 1994). Although the cost of producing a complex repertoire is unknown (Gil & Gahr 2002), the neural pathways associated with song learning and production are well understood (Brainard & Doupe 2000; figure 1). In particular, the high vocal centre (HVC), a telencephalic nucleus associated with the production of complex signals, shows covariation with song complexity both within (Airey *et al.* 2000*a*; Garamszegi & Eens 2004) and between species (DeVoogd *et al.* 1993). It has been suggested that the cost of developing or maintaining the brain nuclei associated with song production could be sufficient to mediate the cost of the trait (Catchpole 1996). Restriction or reallocation of essential resources during early life could therefore compromise the development of the song nuclei (Nowicki *et al.* 1998). This 'developmental stress hypothesis' has been suggested to explain the evolution of complex songs as indicators of male quality (Nowicki *et al.* 1998; Buchanan *et al.* 2003). Recent demonstration that early developmental conditions affect both song quality and quantity, in a range of species, has provided evidence that song may be more condition dependent than previously thought (Nowicki *et al.* 2000, 2002*b*; Buchanan *et al.* 2003; Spencer *et al.* 2003).

To test experimentally the developmental stress hypothesis and the underlying mechanism, we recently demonstrated that male zebra finches (*Taeniopygia guttata*) raised under developmental stress develop simpler, shorter songs than their control foster siblings (Spencer *et al.* 2003). A crucial assumption of the developmental stress hypothesis is that such effects occur through detrimental effects on neural development, which cannot be compensated for in later life. We therefore sought to test whether the detrimental effects of developmental stress on song production seen in the zebra finch were associated with changes in the volumes of the song control nuclei, using a combination of a cross-fostering design and experimental stressors. Here, we detail the results of neural analyses from the same songbirds earlier analysed for differences in song behaviour (Spencer *et al.* 2003). In particular, we hypothesized that the HVC should be differentially susceptible to the detrimental effects of stress, in comparison with other brain nuclei in the song control system.

2. MATERIAL AND METHODS

(a) *Experimental treatments*

Randomly paired zebra finches ($n = 40$ pairs) were assigned to breed and entire clutches of all pairs were randomly cross-fostered during incubation, matched for projected hatch date and clutch

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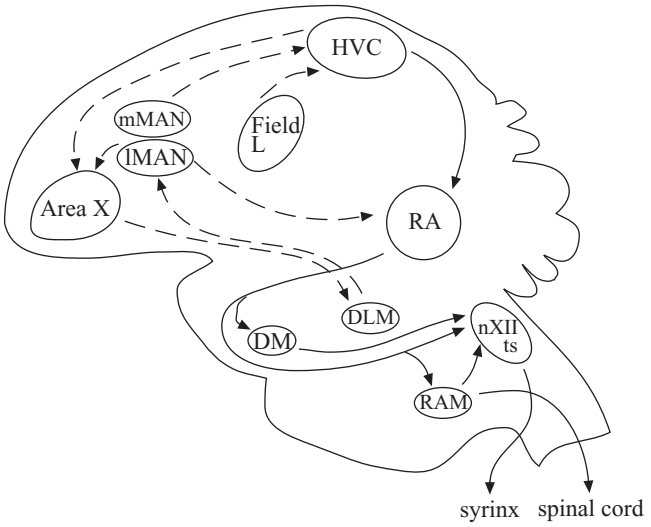

Figure 1. Schematic illustration of main neuronal pathways in the songbird brain. Solid lines indicate the descending motor control pathway for song production; dotted lines show the forebrain recursive loop that is involved in auditory-motor feedback and vocal learning. Abbreviations: DLM: nucleus (n.) dorsolateralis thalami, pars medialis; DM: n. dorsomedialis; HVC: high vocal centre; lMAN, mMAN: lateral and medial parts of n. magnocellularis nidopallii anterioris; nXIIts: n. nervi hypoglossi, pars tracheosyringealis; RA: n. robustus arcopalli; RAM: n. retroambigualis.

size before hatching. Pairs were bred sequentially under control and 'stressed' conditions over two breeding attempts, in a randomized order, such that half of the pairs experienced the stress conditions in their first breeding attempt and half the pairs experienced control conditions during their first breeding attempt. Each pair therefore bred once under control conditions and once under 'stressed' conditions. The 'stress' conditions consisted of one of two types of 'stress':

(i) food restriction during chick feeding, or
(ii) corticosterone administration to the chicks.

Pairs therefore experienced 'control conditions and food stress' or 'control conditions and corticosterone stress', but not both. Under these experimental stress treatments, between 5 and 30 days after hatching, the parents and offspring experienced

(i) food stress: a restricted food supply by mixing the seed with nutritionally valueless husk, in a seed : husk ratio of 1 : 3 to mimic a resource-poor environment (Lemon 1993), or the offspring experienced
(ii) corticosterone stress: daily administration of oral corticosterone dissolved in peanut oil (0.019 mg daily to the age of 10 days and thereafter 0.012 mg daily until day 30).

A pilot study was conducted to calibrate the dose levels within physiological levels. Both control and food-stressed offspring received peanut oil only as a control for this medium. At 30 days after hatching all offspring were moved to *ad libitum* food conditions and exposure to peanut oil and corticosterone was discontinued. To ensure that the treatments did not affect the song output of the fathers and therefore song tutoring, we recorded the breeding males at least twice for 20 min during the treatment period and found no difference on mean bout rate between treatments ($F_{1,24} = 1.32$, $p = 0.287$) (Spencer *et al.* 2003). From day 35 until day 60 post-hatching the offspring were kept within visual and acoustic contact of their parents, but separated by wire mesh. At 60–70 days the young birds were moved to sex-specific aviaries. At this time their parents were given food *ad libitum* for 8–10 days before starting the second breeding attempt. The song output of the birds from this experiment was also recorded and the effects of these treatments on their song behaviour are documented elsewhere (Spencer *et al.* 2003). The songs from all the breeding males were analysed for motif duration (seconds), number of syllables in motif, number of different syllables in motif and peak frequency of motif (kHz) (Spencer *et al.* 2003). All experimental procedures were carried out under the Animals (Scientific Procedures) Act 1986, and were regulated by the Home Office of HM Government, UK.

(b) *Neural analyses*

At a mean age of 215 ± 10 (s.e.m.) days old, male offspring were weighed and then killed by means of an overdose of chloroform. The birds were perfused transcardially with 0.9% saline followed by 4% phosphate-buffered formaldehyde solution. Brains were post-fixed and their mass was recorded. At this time the testes were also removed and weighed. One half of each brain was immersed in RNAse-free 10% phosphate-buffered sucrose, followed by 30% phosphate-buffered sucrose. Brain halves were then sectioned parasagittally on a freezing microtome at 40 μm, collected in phosphate-buffered saline and mounted onto Superfrost Plus microscope slides for both Nissl staining and androgen receptor (AR) detection. Expression of AR mRNA in the brain sections was detected with cRNA probes labelled with cytidine 5′-(α-thio)triphosphate (^{35}S-CTP) of the zebra finch using an *in situ* hybridization protocol (Metzdorf *et al.* 1999). Slides were analysed under brightfield (Nissl) and darkfield illumination (AR) with a Leitz Aristoplan microscope (Leitz Wetzlar, Germany). The areas of brain regions HVC, robust nucleus of the arcopallium (RA) and lateral magnocellular nucleus of anterior nidopallidum (lMAN) (Reiner *et al.* 2004) were video-digitized using a PC equipped with an image analysis system (MetaMorph 4.6, Visitron Systems, Germany). Volumes were calculated from every third section as the sum of the area sizes multiplied by 120 μm (section interval × section thickness) for Nissl and AR, respectively.

(c) *Statistical analysis*

All statistical analyses were conducted using ANOVA (Minitab 13). The effects of the experimental treatments on the volumes of the brain nuclei were tested using a general linear model for each brain variable. Brood order (first or second brood) and experimental treatment were entered as fixed factors and morphological variables relating to offspring growth and development were entered as covariates. Foster environment was nested within brood order, where order is whether the stress treatment occurred during either the first or second breeding attempt. This analysis allowed us to compare the brain development of pairs of unrelated male foster siblings, raised by the same foster father, in either stressed or control conditions. This is important, as this paired analysis allows comparison of unrelated offspring from sequential breeding attempts which have had the same parental care and tutoring experience. All the models examining neural development therefore compare the brain structures of unrelated foster siblings raised in sequential breeding attempts by the same foster parents. Data detailing neural development were available for male foster offspring from successive breeding attempts of 16

Table 1. Factors describing variation in brain morphology of adult male zebra finches raised under experimental or control conditions.
(Experimental treatments were food deprivation and corticosterone administration. Brood order refers to whether first or second broods experienced a stress treatment and foster environment refers to the identity of the foster parents. All broods were cross-fostered before hatching. $p > 0.05$ = n.s.; $0.05 > p > 0.01$ = *; $p < 0.01$ = **.)

	brain morphology									
	brain weight		RA		lMAN		HVC (Nissl stain)		HVC (androgen receptor)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
experimental treatment	0.26	n.s.	0.42	n.s.	0.71	n.s.	6.96	*	61.19	**
brood order	1.98	n.s.	0.00	n.s.	0.44	n.s.	20.38	**	128.52	**
foster environment (brood order)	4.43	**	2.97	*	6.87	**	4.10	*	42.51	**
fledging mass					5.11	n.s.	2.49	n.s.	130.55	**
body mass 100 days					5.32	n.s.	9.09	*	84.16	**
fledging mass × body mass 100 days							7.38	*		
experimental treatment × brood order									69.62	**
brain mass									1365.25	**
relative mass of testes									36.82	**

pairs of parents. In all cases but one, only one male per brood was analysed, with a mean from two male offspring taken in one case. Stepwise deletion of non-significant terms was performed until the minimal adequate model was found. The model residuals were checked for normality and homoscedasticity at each step. Non-significant terms were retained only when they hovered below significance and appeared to be biologically meaningful.

3. RESULTS

Comparing broods raised by the same foster parents, we found that the foster environment had a significant effect on all brain variables (table 1). Confirming the plasticity of the brain as regards early developmental conditions, we found that foster siblings raised by the same foster parents in successive broods were significantly more similar to each other in terms of adult brain weight and the volume of adult brain nuclei (HVC (both Nissl- and androgen receptor-delineated), RA and lMAN) than to other random individuals. As foster siblings are unrelated, this suggests strong environmental effects on brain development, mediated either through the quality of parental care or the song behaviour of the foster father.

Both of our experimental stress treatments (food restriction and corticosterone administration) resulted in a significantly decreased volume of the HVC versus the controls, while other brain variables were not affected (figure 2, table 1). The direction of the treatment effects on HVC (Nissl) were identical to the results shown for HVC (androgen receptors) in figure 2. We also found significant positive effects of body mass at fledging and at maturity, and an interaction between these two measures, on HVC development (table 1), confirming that chicks that had higher body masses as juveniles had larger HVC volumes as adults. Together, these results confirm the fundamental condition dependence of HVC development and illustrate how resource allocation to neural development could be compromised in adverse conditions. The significant brood order effect found for HVC size indicated that pairs that bred first under control and then under stressed conditions produced offspring with smaller HVCs than parents that bred under stressed and then control conditions. Other variables affecting HVC size (via androgen receptor delineation) were fledging mass and relative size of the testes, which both had negative effects, while body mass at 100 days and brain mass related positively to HVC size.

To investigate further the effects of rearing environment on brain development we tested whether any variables related to the song of the genetic and foster father significantly explained individual variation in offspring HVC size. The results demonstrated that significant variation in offspring HVC size was described only by the number of syllables in the song of the genetic father ($F_{1,26} = 6.86$, $p = 0.014$) and the number of syllables in the song of the foster father ($F_{1,26} = 5.66$, $p = 0.025$), as well as their interaction ($F_{1,26} = 5.98$, $p = 0.022$). In both cases, parental song complexity had a positive effect on offspring brain development (coefficients; foster father = 0.124, genetic father = 0.140), although the interaction indicates that the slopes of these positive effects are significantly different.

4. DISCUSSION

Our results not only demonstrate that the HVC is vulnerable to developmental stress effects, but suggest that it is more susceptible to developmental stress than other brain nuclei or overall brain mass. This may explain the mechanism underlying the evolution of song as an honest indicator of quality (Catchpole & Slater 1995; Nowicki *et al.* 1998, 2002*a*).

The present results suggest that the positive relationship between HVC size and both song repertoire size and phrase duration, found previously in the zebra finch (Airey & DeVoogd 2000, although see MacDougall-Shackleton *et al.* (1998)), occurs in part as a result of genetic control and in part as a result of environmental conditions. Cross-fostering suggests the importance of both genetic control and environmental exposure during the song-learning period, although a partial cross-fostering design is needed

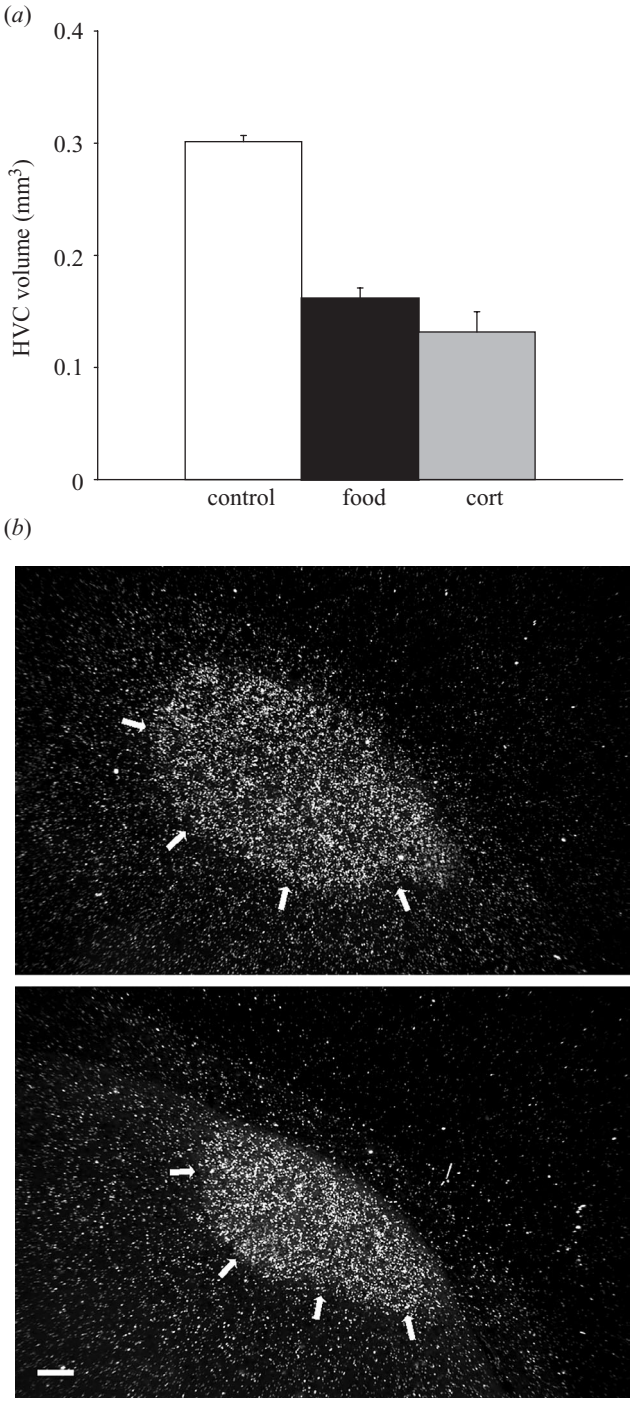

Figure 2. (*a*) Volume of the HVC as delineated by androgen receptors for male zebra finches raised in the three treatment groups. White bar, control group; black bar, food restriction group; grey bar, corticosterone administration group. The directions of the effects on HVC (Nissl data) are identical to those shown here. Fitted means + s.e.m. from the general linear ANOVA model are given. (*b*) Darkfield photomicrographs of the HVC nucleus of a male zebra finch from the control group (top) and the corticosterone stress group (bottom) showing androgen receptor mRNA expression. Arrows indicate the borders of the HVC. Scale bar, 100 μm.

to quantify the relative importance of these effects. Previous work on zebra finch neural development has confirmed the importance of auditory experience during brain development (Ward *et al.* 1998). To date, the results of studies testing the heritability of the size of such nuclei have suggested a genetic component (Airey *et al.* 2000*b*), although such studies have failed to separate genetic and environmental effects adequately.

Plasticity in HVC development appears to be partly a result of an increased presence of oestrogen receptors in this nucleus (Gahr & Konishi 1988), which play an important role in masculinization of the brain (Gahr 1996). Recently, it has been shown that oestrogenic regulation of androgen receptors is involved in subsequent differential action of androgens in the song nuclei (Kim *et al.* 2004). Consequently, in this study we delineated the HVC by means of androgen receptor distribution (figure 2*b*), according to its projections to the descending motor pathway (Bottjer & Johnson 1997). Previous work has shown that siblings appear to have very similar degrees of HVC development, in terms of cell number (Ward *et al.* 2001), although it remains unclear whether these effects are the result of genetic effects, maternal effects or differences in song stimulation during development. The current results suggest important roles for both environmental and genetic control of a sexual signal, and these controls are likely to influence individual fitness (Andersson 1994). There were strong effects of brood order on HVC development, such that pairs that bred first under control and then under stressed conditions produced male foster offspring that had significantly smaller HVC volumes than pairs that bred first under stressed and then under control conditions. Such effects may be the result of changes in parental care or tutoring regimes across broods. If zebra finch offspring from second breeding attempts receive lower-quality care or investment than those in first broods, stressful conditions during the second breeding attempt may have caused more detrimental effects than during the first breeding attempt. With respect to male tutoring behaviour, it is worth noting that in a comparison of male song output, no treatment effects on song bout rate were found (Spencer *et al.* 2003).

Our results suggest that HVC size is particularly vulnerable to the effects of developmental stress, but they do not reveal the mechanism behind this effect. Corticosteroid receptors, which are known to be present in passerine brains and are seasonally regulated (Breuner & Orchinik 2001), may play a role. Alternatively, cells within the HVC may be selectively vulnerable to the effects of oxidative stress and damage through free radicals (von Schantz *et al.* 1999). Our treatment results suggest that corticosterone could mediate the effects of developmental stress, although it is worth noting that there was no detectable rise in circulating corticosterone levels within the birds in our 'food deprivation' group (Spencer *et al.* 2003), suggesting that the effects of stress may occur through multiple routes. We suggest that the results detailed here demonstrate that the developmental stress hypothesis could explain the evolution of complex repertoires through selective control of HVC size. Alternative hypotheses explaining the evolution of complex repertoires suggest that such traits may act as honest indicators of quality, because they are associated with increased energetic costs, physical constraints on the production of complex sounds, or because song complexity is age-related (Andersson 1994; Gil & Gahr 2002). In our recent test of the developmental stress hypothesis in the European starling (*Sturnus vulgaris*) we found strong evidence in support of the role of early energetic restriction mediating the costs of song development, without invoking

age-related changes in repertoire complexity (Buchanan *et al.* 2003). The developmental stress hypothesis has been substantiated by the finding that developmental stress affected the accuracy of song copying in male song sparrows (Nowicki *et al.* 2002*b*), with associated changes in overall brain size and in relative volume of the RA (Nowicki *et al.* 2002*a*). Here, we found that significant variation in the complexity of both the foster and genetic fathers' songs explained variation in HVC development in the offspring brain, confirming that both genetic and environmental factors affect brain structure. We suggest that this input takes the form of song tutoring from the foster father, as the model for song learning has an important influence on the final song structure (Eales 1985). However, the standard of parental care will also play an important role in determining the resources available to invest in neural growth. In terms of the genetic input, our data suggest that there is a strong genetic contribution determining HVC size and therefore repertoire complexity. Such genetic determination, which may involve genes allowing differing resistance to environmental stress, would be necessary to invoke a handicap argument to explain the evolution of song complexity as a sexually selected trait (Andersson 1994).

Here we demonstrate for the first time, to our knowledge, the differential vulnerability of the HVC, the brain area principally associated with the production of complex songs, to the effects of developmental stress. In demonstrating the importance of developmental stress in a species with a sexually selected song structure, these results also provide fundamental information on the mechanisms underlying the evolution of complex songs as honest indicators of male quality.

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