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ENVIRONMENTAL AND GENETIC CONTROL OF BRAIN AND SONG STRUCTURE IN THE ZEBRA FINCH

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Birdsong is a classic example of a learned trait with cultural inheritance, with selection acting on trait expression. To understand how song responds to selection, it is vital to determine the extent to which variation in song learning and neuroanatomy is attributable to genetic variation, environmental conditions, or their interactions. Using a partial cross fostering design with an experimental stressor, we quantified the heritability of song structure and key brain nuclei in the song control system of the zebra finch and the genotype-by-environment (G × E) interactions. Neuroanatomy and song structure both showed low levels of heritability and are unlikely to be under selection as indicators of genetic quality. HVC, in particular, was almost entirely under environmental control. G × E interaction was important for brain development and may provide a mechanism by which additive genetic variation is maintained, which in turn may promote sexual selection through female choice. Our study suggests that selection may act on the genes determining vocal learning, rather than directly on the underlying neuroanatomy, and emphasizes the fundamental importance of environmental conditions for vocal learning and neural development in songbirds.

KEY WORDS: **Birdsong, genotype-by-environment interaction, heritability, HVC, song system, *Taeniopygia guttata*.**

Understanding the causes of phenotypic variation in complex traits is essential in order to explain how genetic variation is maintained, despite selection on the trait (Houle 1992). To determine how complex vocal communication has evolved in birds, it is crucial that we quantify the genetic variation underlying the expression of this trait and the impact of environmental quality on this variation (Hoffmann and Merila 1999), as well as any interactions between them (Buchanan et al. 2013). Such genotype-by-environment (G × E) interactions are important because they demonstrate the reaction norms of genotypes in relation to changes in environmental conditions and determine whether an ornament can signal heritable quality (Cotton et al. 2004). Although the relative impact of genetic and environmental influences on avian vocal learning has been tested repeatedly over the last 40 years, virtually no studies have investigated these effects in relation to the neural control of song.

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It has been proposed that birdsong is an honest signal of early developmental conditions, which mediate the development of song control nuclei in the oscine forebrain (Nowicki et al. 1998; Buchanan et al. 2003). If so, song could act as a signal of developmental stability and therefore phenotypic quality (Nowicki et al. 1998, 2002), because developmental stress has long-term effects on fitness (including morphology, fecundity, quality of offspring, antioxidant defenses, and potentially longevity: Birkhead et al. 1999; Metcalfe and Monaghan 2001; Blount et al. 2003; Metcalfe and Monaghan 2003; Arnold et al. 2007). Although there is support for this developmental stress hypothesis (Spencer and MacDougall-Shackleton 2011; MacDougall-Shackleton and Spencer 2012), prior studies have failed to take into account that phenotypic expression of song may arise from G × E interactions (Swaddle 2011; MacDougall-Shackleton and Spencer 2012). Different genotypes may differ in the robustness of their development when facing a given level of environmental challenge (Andersson 1994; Nowicki et al. 1998; Swaddle 2011). Under certain conditions, G × E interactions may help maintain additive genetic variation in male secondary sexual traits and are predicted to promote female choice (Kokko and Heubel 2008), particularly if there is little variation in the environmental conditions experienced by males. In other circumstances, particularly where there are high dispersal rates or very high levels of G × E interaction, traits are unlikely to be subject to strong, direct sexual selection, due to the unreliability of the signal across different environments (Greenfield and Rodriguez 2004; Kokko and Heubel 2008; Higginson and Reader 2009). As G × E interactions have been documented for a number of secondary sexual characteristics (Wilkinson 1987; Jia et al. 2000; Qvarnström et al. 2000; Danielson-Francois et al. 2006), they have important implications not only for trait expression, but also for female preferences. This study is the first to quantify both song and brain structure in terms of G × E.

The avian song control system is characterized by high levels of developmental plasticity (Brenowitz and Beecher 2005). Indeed, plasticity extends into adulthood in every species examined to date and seems to be the norm (Brenowitz and Beecher 2005). The size and structure of song control nuclei are influenced by seasonal variation (Brenowitz and Beecher 2005), auditory experience (Brainard and Doupe 2002), exposure to gonadal (Brainard and Doupe 2002) and glucocorticoid hormones (Buchanan et al. 2004), and the developmental environment (Nowicki et al. 2002; Buchanan et al. 2004). Given this high level of developmental plasticity, one would expect to find only low levels of heritability in the song system. Surprisingly however, the best estimates so far of the heritability of song control nuclei in the oscine brain come from Airey et al. (2000a), and suggest that the volume of HVC (used as a proper name, Reiner et al. 2004), and the robust nucleus of the arcopallium (RA), along with overall brain and body mass, are moderately heritable in the zebra finch, *Taeniopygia guttata* (heritability estimates, h^2: body mass = 0.32; brain mass = 0.49; HVC volume = 0.38; RA volume = 0.72; Airey et al. 2000a). Other studies have suggested that the number of HVC neurons (Ward et al. 2001) and the rate of neurogenesis (Hurley et al. 2008) in the HVC are predicted by genetic origin. In humans, brain volume is highly heritable (reviewed in Peper et al. 2007), as are many specific brain regions (Peper et al. 2007; Kremen et al. 2010). It should also be noted, though, that common environment and unique environmental conditions also explain much of the individual variation in volume of several brain regions in humans (Peper et al. 2007; Kremen et al. 2010).

There are several reasons to suspect that these surprisingly high heritabilities have been overestimated and do not reflect realistic estimates for wild songbirds. These studies employed laboratory bird populations, raised under controlled conditions, which are likely to have experienced unusually low levels of environmental variation resulting in low levels of phenotypic variation. The phenotypic variation that can be attributed to genetic differences thus accounts for a higher than expected proportion of the total observed phenotypic variation, inflating the estimated heritability estimates. In addition, none of these studies attempted to determine the effects of G × E interactions on brain development. Finally, and crucially, birds were raised and tutored by their genetic parents in all three studies, making it impossible to separate heritable variation from the effects of a common rearing environment, which are expected to be significant as birdsong is learned from a tutor (Marler 1990; Catchpole and Slater 2008).

Forstmeier et al. (2009) used pedigree-based animal models to calculate the heritability of a number of call and song characteristics in a captive population of 808 zebra finches. They found that unlearned female calls were more heritable than male song. Male songs showed a greater component of environmental variance than did female calls, and structural song traits, such as syllable number or song phrase duration had low estimates of heritability (h^2: syllable number = 0.11; phrase duration = 0.18; Forstmeier et al. 2009). All these results are consistent with the interpretation that the song control nuclei in the brain are susceptible to environmental challenges and that learned song represents the combined influences of environment, genes, and their interaction.

In this study, we used a partial cross-fostering design to tease apart the contributions of genotype, the rearing and learning environment and nutritional restriction to variation in brain development and adult song between zebra finches. An experimental nutritional restriction treatment was employed to induce greater variance in the environmental conditions experienced by our subjects, by comparison to prior studies. Thus, we were able to determine the response of different genotypes, within and across family groups, in relation to developmental conditions. This design allowed us, for the first time, to estimate the heritability of

neural traits associated with song without the confounding factors of the tutoring and rearing environment, as well as to quantify G × E interactions. Specifically, we sought to test whether any trait showed a significant G × E interaction, which would indicate the capacity of some genotypes to withstand the effects of environmental challenges more than others and indicate the potential for traits to be used as reliable indicators of individual quality.

Methods

BIRDS AND EXPERIMENTAL TREATMENTS

In 2006, pairs of zebra finches from the breeding colonies at the Max Planck Institute for Ornithology, Seewiesen, Germany (identified as population 15, Seewiesen-NL, by Forstmeier et al. 2007), were allocated randomly to a breeding cage (40 × 40 × 40 cm) under a 12:12 light/dark photoperiod. All breeding pairs were within earshot of each other but not in visual contact. All breeding pairs were allowed access to a nestbox and nest material and the progress of the reproductive attempt was followed daily. Each pair produced four broods, two under control conditions and two under nutritional stress, in randomized order. At day 1–3 posthatching, a randomly chosen half of each brood were cross-fostered to a brood of the other treatment group of similar age. Broods that did not have a suitably aged counterpart for cross-fostering were removed from the experiment. Brood sizes were not altered. Between days 5–30 post-hatch broods were fed ad lib seed alone (control) or a restricted amount of seed (nutritional stress), adjusted for age and brood size, mixed 2:1 with husks which offered no nutritional benefit and increased foraging times for a given amount of seed (Woodgate et al. 2010). At day 30, all nestlings were moved onto an ad lib diet of seed with green vegetables. At day 60 posthatching, nestlings were removed to a cage (40 × 40 × 40 cm) adjacent to, and sharing visual and acoustic contact with, their home cage. After 80 days all nestlings were transferred to a large sex-specific aviary with large outdoor (416 × 242 cm and 302 cm high) and indoor (403 × 301 cm and 200 cm high) compartments. Nestlings were weighed at the end of the nutritional restriction, 30 days posthatch, and in adulthood. Fathers and foster-fathers were weighed once their final brood reached adulthood.

In total 107 broods were produced, 56 under control and 51 under food restricted conditions. A total of 169 males and 152 females were produced in this experiment (mean brood size [number of chicks hatched in each brood] ± 1 SD: control broods: 3.0 ± 1.1; restricted broods: 3.0 ± 1.0). Not all breeding pairs produced adult males originating from all four combinations of fostering and food restriction treatment, so a reduced sample size of 48 males was available for this study. These were the offspring of 18 different genetic fathers and were drawn from 12 breeding pairs (six of which raised control then food restricted broods and six of which raised food restricted then control broods). Four males reared by each breeding pair were selected: one genetic- and one foster-son from control broods and one genetic- and one foster-son reared under the food restriction treatment. When more than one male was available from each combination of treatments, the subjects were selected at random.

CORTICOSTERONE ASSAYS

A blood sample was taken from each bird in the first two broods at day 30 posthatch, to measure plasma corticosterone levels. Blood samples were taken within 1 min of the experimenter entering the room and no two birds from the same cage were sampled on the same day. Corticosterone concentration was determined by direct radioimmunoassay (RIA) following Goymann et al. (2006). Mean ± SD extraction efficiency for plasma corticosterone was 87.2 ± 3.7%. Standard curves and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc., Fullerton, CA), using a four parameter logistic curve fit. The lower detection limit of the standard curves was calculated as the first value outside the 95% confidence intervals for the zero standard (B_{max}) and was 24.2 pg/mL. Intraassay coefficients of variation were 6.4% and 5.2%. The interassay variation was 1.1%.

SONG RECORDING AND ANALYSIS

Offspring males were transferred to individual recording cages together with an unfamiliar female (selected at random from the breeding colony), at 169 (± 40) days old. Fathers' and foster fathers' songs were recorded at the end of the breeding period in, together with their mate. Recordings were made using a Sennheiser ME67 directional microphone (Sennheiser, Wedemark, Germany) and a Sony TCD-5M tape recorder. All recordings were made at a standardized distance, with no background noise. At least 20 songs were recorded from each male. The recordings were digitized using Canary 1.2.1 software (Cornell Laboratory of Ornithology, Ithaca, NY) on a Power Macintosh 7200/90. The sampling rate was 22 kHz with a 16 bit sample size, and the frequency/time resolution was 342 Hz with an FFT size of 256 points. Song recordings were analyzed as previously described in Woodgate et al. (2012) using Raven 1.2 sound analysis software (Cornell Laboratory of Ornithology). The 5 song parameters measured were "syllable number" (the total number of syllables in the song phrase, excluding introductory syllables), "phrase length" (duration of the song phrase in seconds), "peak frequency" (the frequency at which the maximum power in the phrase occurs, measured in kHz and determined using Raven's built-in function), "maximum frequency" (the highest frequency reached in the song phrase, measured from the spectrogram in kHz), and "proportion unique syllables" (the number of distinct syllable types that make up the song phrase, expressed as a proportion of the total number of syllables).

BRAIN MEASUREMENTS

At 169 (± 40) days, the male offspring and their fathers and foster-fathers were killed by decapitation and their brains removed immediately by dissecting them out of the skull. The brains were weighed using a Sartorius BA110S balance (Sartorius AG, Goettingen, Germany). After dissection, brains were immediately frozen on dry ice and stored at −80°C until analysis. Brains were sectioned at 30 μm using a Leica CM 3050S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted sections were stained with thionin for Nissl staining. The area of HVC and RA in every third section were measured by digitizing every third section using a PC equipped with MetaMorph 4.6 image analysis system (Visitron Systems, Germany). HVC and RA volumes were calculated the sum of the areas multiplied by section thickness, as described in Leitner and Catchpole (2002).

STATISTICAL ANALYSIS

Statistical tests were carried out using SPSS 20 (IBM Corporation, Armont, NY). To test the relative contributions of genetic and environmental factors in determining the phenotypes of males, we ran a series of father–son regressions. Each brain, body, and song variable was analyzed as the dependent variable in a linear mixed model which included the corresponding value of the same trait from both their genetic father and the rearing male as covariates (hereafter referred to as the "genetic father's phenotype" and "rearing male's phenotype"). There is evidence that both brood size (Soma et al. 2006) and the number of male nest-mates to which a young bird is exposed (Tchernichovski and Nottebohm 1998; Gil et al. 2006) can affect song development, so we also included the number of male siblings (or foster-siblings) in the nest and the brood size as covariates. The rearing male was the genetic father of all nonfostered offspring and the foster-father of those in the cross-fostering treatment. The initial model included nutritional treatment, cross-fostering treatment, and brood number as fixed factors along with the two-way interaction terms "nutritional treatment by rearing male's phenotype" and "nutritional treatment by genetic father's phenotype." This initial model was simplified by the sequential elimination of nonsignificant interactions and terms, with the final model constrained to retain the main effects of nutritional treatment and the genetic father's and rearing male's phenotypes. The number of male nest-mates and the brood size were dropped from all the final models except that for HVC volume. Table S3 presents a series of alternative models in which number of males and brood size were retained. The results of these models are not qualitatively different from the analyses presented in Table 1 and the estimates they yield of h^2 are extremely similar (differing by no more than 0.06). There were no significant effects of cross-fostering or brood number so both terms were dropped from the final models. The heritability (h^2) was estimated as twice the slope of the father–son regression. It should be noted that this method unavoidably includes some pseudoreplication, as the same parental males acted as rearing or genetic father to several offspring.

Because of this, we employed a second method of estimating heritabilities, this time using sib–sib comparisons. We estimated the genetic, environmental, and G × E components of the variation in body, brain, and song variables, (Merila 1996; Christe et al. 2000). GLMs were used to calculate these variance components using restricted maximum likelihood estimates. The model included nutritional treatment as a fixed factor, nest of genetic origin and rearing nest as random effects, and the interaction term between nutritional treatment and genetic origin. The total phenotypic variance for each trait (V_P) was calculated as $V_P = V_A + V_{GE} + V_{EC} + V_E$. We estimated the additive genetic variance (V_A) as double the variance due to nest of genetic origin. Nest of genetic origin estimates $\frac{1}{2}V_A$ but also includes one quarter of the dominance variance (V_D) and any variation attributable to maternal effects (V_M), if present. The term "rearing nest" estimates variance due to effects of the common environment (V_{EC}), including variation in parental care, song tutoring, etc. The nutritional treatment by genetic origin interaction represents part of the variance due to G × E interactions (V_{GE}). Finally, the error component of variance includes any environmental effects (V_E) not attributable to common rearing environment (including any effect of the nutritional treatment) and the remaining portion of V_{GE} not due to nutritional treatment by genotype interaction, as well as $\frac{1}{2}V_A$ and $\frac{3}{4}V_D$. Heritability was calculated as $h^2 = V_A/V_P$. We also calculated the proportion of phenotypic variance attributable to a common rearing environment (V_{EC}/V_P), and to nutritional treatment by genotype interaction (V_{GE}/V_P). The coefficient of variation for phenotypic variation (CV_P), additive genetic variation (CV_A), variation due to common rearing environment (CV_{EC}) and variation due to nutritional treatment by genotype interaction (CV_{GE}) were calculated as $CV = 100\sqrt{V}/\bar{X}$, where $\bar{X}$ is the mean value for each trait (Charlesworth 1984; Houle 1992). We used a jack-knifing procedure to generate a resampling distribution of the variance components, and used this distribution to calculate heritability estimates and their standard errors. Heritability estimates were considered statistically significant if they were more than two standard errors greater than zero (Merila 1996).

We investigated the effect of the nutritional treatment on nestling mass at day 30 posthatching (the end of the experimental manipulation and point of nutritional independence), using general linear models (GLMs). Male ($N = 36$ control, 34 restricted) and female ($N = 39$ control, 39 restricted) nestling mass was analyzed separately using a model in which nutritional treatment was the fixed factor and nest of genetic origin and rearing nest were included as random factors. Effect sizes (d) were calculated for significant effects as the difference between the means divided by the pooled standard deviation. We also tested for an effect of the

Table 1. Results of father–son regressions testing the effects of nutritional restriction, genetic factors, and common rearing environment on 9 body, brain, and song variables. Covariate terms are indicated in italics. Significant *P* values are indicated in bold.

Dependent variable	Model term	F	df	P	Slope ± SE	h^2
Body mass	Nutrition	0.01	1,44	0.919		
	Genetic father	3.65	1,44	0.063	0.31 ± 0.15	0.62
	Rearing male	1.41	1,44	0.241	0.18 ± 0.15	
Brain mass	Nutrition	5.42	1,43	**0.025**		
	Genetic father	0.10	1,43	0.668	−0.05 ± 0.20	−0.11
	Rearing male	0.04	1,43	0.849	−0.03 ± 0.15	
	Nutrition × Genetic father	5.49	1,43	**0.024**	0.34 ± 0.16	
HVC volume	Nutrition	0.12	1,43	0.734		
	Genetic father	0.21	1,43	0.646	0.11 ± 0.23	0.21
	Rearing male	0.03	1,43	0.857	0.04 ± 0.22	
	Male nest-mates	6.00	1,43	**0.018**	0.05 ± 0.02	
RA volume	Nutrition	0.65	1,44	0.424		
	Genetic father	3.56	1,44	0.066	0.38 ± 0.19	0.76
	Rearing male	1.45	1,44	0.235	−0.23 ± 0.19	
Syllable number	Nutrition	5.10	1,42	**0.029**		
	Genetic father	0.68	1,42	0.415	0.12 ± 0.14	0.23
	Rearing male	4.22	1,42	**0.046**	0.46 ± 0.23	
Phrase length	Nutrition	1.27	1,42	0.266		
	Genetic father	0.01	1,42	0.919	0.02 ± 0.16	0.03
	Rearing male	<0.01	1,42	0.949	0.02 ± 0.24	
Peak frequency	Nutrition	4.77	1,42	**0.035**		
	Genetic father	0.42	1,42	0.519	−0.09 ± 0.14	−0.18
	Rearing male	1.28	1,42	0.264	0.14 ± 0.13	
Maximum frequency	Nutrition	3.62	1,42	0.064		
	Genetic father	2.41	1,42	0.128	0.28 ± 0.18	0.56
	Rearing male	0.23	1,42	0.634	0.08 ± 0.17	
Proportion unique syllables	Nutrition	5.84	1,41	**0.020**		
	Genetic father	2.81	1,41	0.101	0.23 ± 0.14	0.47
	Rearing male	7.81	1,41	**0.008**	0.06 ± 0.19	
	Nutrition × Rearing male	6.53	1,41	**0.014**	0.67 ± 0.26	

nutritional treatment on circulating corticosterone levels (measured in pg/ml of blood) in nestlings from the first and second broods at day 30. A Box–Cox transformation ($\lambda = 0.2$) was used to normalize the corticosterone data which was analyzed using the same models as were used to analyze nestling mass.

Results

HERITABILITY OF PHENOTYPIC TRAITS

The relative contributions to phenotypic variance of genetic origin, common rearing environment, and interaction between genotype and the nutritional treatment are shown in Figure 1. Father–son regressions indicated that the genetic father's phenotype did not significantly predict that of the son for any variable, although there were nonsignificant trends toward body mass and RA volume being heritable (Table 1). By contrast, analysis of the variance components in the sib–sib comparison indicated that body mass was highly heritable (Table 2 and Fig. 1), as was maximum frequency, whereas RA volume (Table 2 and Fig. 1) and syllable number were moderately heritable (Table 2 and Fig. 1). Although nonsignificant, the heritability estimates from the father–son regression were largely in agreement with those derived from variance components between siblings, with the exception of HVC volume (Tables 1, 2).

Both analytical approaches consistently show a significant interaction between the experimental treatment and genotype in determining sons' brain mass (Tables 1, 2). The reaction norm of brain mass therefore varies across nutritional environments, suggesting that there is no one genotype which always produces a large brain. Repeating the father–son regressions for nutritionally restricted and control sons separately showed that there was no link between the brain mass of genetic fathers and their sons in the control group ($F_{1,21} = 0.79$, $P = 0.384$, slope of regression = 0.25), whereas there was a nonsignificant trend toward a negative

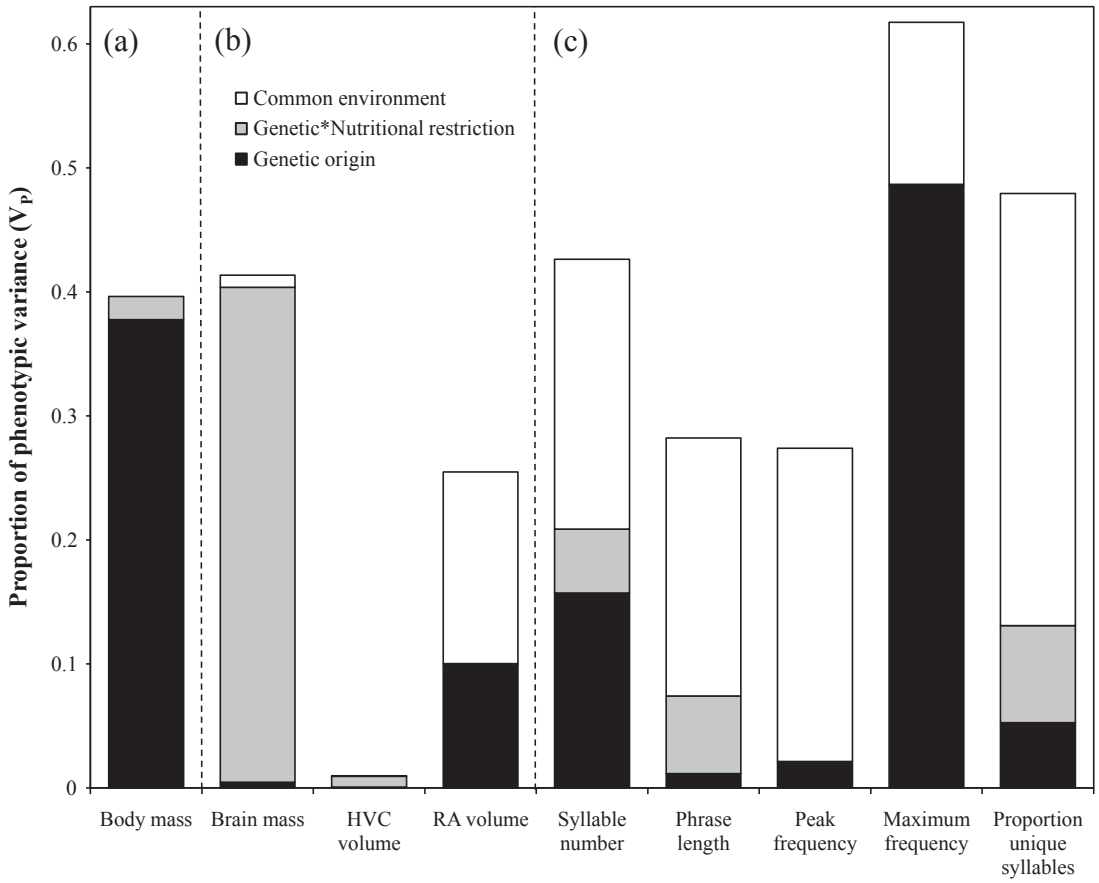

Figure 1. **Causal components of variance in (a) body mass, (b) brain structure, and (c) song traits. Variance components were estimated using restricted maximum likelihoods and are expressed as a proportion of the total phenotypic variance (V_P). The error variance (not shown) accounts for the remainder of the variance for each trait. Note that the effect of nest of genetic origin estimates $^1/_2V_A$.**

relationship between the brain mass of fathers and their sons in the nutritional treatment ($F_{1,21} = 3.23$, $P = 0.087$, slope of regression $= -0.38$)

EFFECTS OF REARING ENVIRONMENT ON PHENOTYPE

Father–son regressions suggested that the rearing male's song phenotype predicted that of sons for syllable number and proportion of unique syllables in a song phrase, but not for the other song variables or for body mass or brain traits (Table 1). Using the sib–sib method, common rearing environment explained a significant proportion of the phenotypic variance in all five song variables, ranging from 0.13 to 0.35 (Table 2 and Fig. 1). Common rearing environment also explained 15% of the variance in sons' RA volume (Table 2 and Fig. 1), but did not account for any of the variance in overall brain mass, HVC volume, or body mass.

There was an interactive effect of nutritional treatment by the proportion of unique syllables in the rearing male's song on sons' proportion of unique syllables (Table 1), demonstrating that song complexity is a function not only of tutoring regime, but of environmental quality. Analyzing the control and nutritionally restricted groups separately showed that the proportion of unique syllables in foster-father's songs predicted that of their sons in the control group but not that of food restricted sons (controls: $F_{1,21} = 16.35$, $P = 0.001$, slope of regression $= 0.71$; restricted: $F_{1,19} = 0.18$, $P = 0.673$, slope of regression $= 0.10$).

EFFECTS OF FOOD RESTRICTION TREATMENT ON PHENOTYPE

Father–son regressions showed that there was no effect of the nutritional restriction on adult body mass, any brain variables, phrase length, or maximum frequency (all $P > 0.05$; Table 1). Males reared under the food restricted treatment had a greater number of syllables in their song than did controls (Table 1, estimated marginal means $\pm$ SE: controls $= 6.44 \pm 0.50$; restricted $= 8.12 \pm 0.53$) and had a higher peak frequency (Table 1, estimated marginal means [kHz] $\pm$ SE: controls $= 2.87 \pm 0.20$; restricted $= 3.49 \pm 0.20$).

The nutritional restriction treatment was successful in manipulating female, but not male, nestling growth rate. At day 30, there

Table 2. Phenotypic (V_P), additive genetic (V_A), common rearing environment (V_{EC}), genotype-by-nutritional-treatment (V_{GE}), and environmental (V_E) components of variance for 9 body, brain, and song variables. Also shown are estimates of heritability (h^2); the proportion of variation attributable to common rearing environment (V_{EC}/V_P), and to nutritional treatment by genotype interactions (V_{GE}/V_P); and the coefficients of variation for each of these factors. Each column shows the mean ± SE, derived from the jack-knifing procedure, for each component. Significant values of h^2, V_{EC}/V_P, and V_{GE}/V_P are indicated in bold type.

Dependent variable	V_P	V_A	V_{EC}	V_{GE}	V_E	h^2	V_{EC}/V_P	V_{GE}/V_P	CV_P	CV_A	CV_{EC}	CV_{GE}
Body mass	2.57 ± 0.07	1.94 ± 0.22	<0.01 ± <0.01	0.05 ± 0.14	1.55 ± 0.12	**0.75** ± 0.08	<0.01 ± <0.01	0.02 ± .06	10.53	9.15	<0.01	1.44
Brain mass	1265.56 ± 46.57	11.16 ± 42.17	12.32 ± 37.68	505.38 ± 87.15	742.28 ± 61.51	0.01 ± 0.04	0.01 ± 0.03	**0.40** ± 0.06	7.87	0.74	0.78	4.97
HVC volume	6227.85 ± 169.88	8.23 ± 46.13	2.54 ± 17.41	54.85 ± 376.04	6216.35 ± 413.95	<0.01 ± 0.01	<0.01 ± <0.01	0.01 ± 0.06	20.87	0.76	0.42	1.95
RA volume	2630.68 ± 96.74	525.19 ± 172.92	406.90 ± 100.81	0.71 ± 4.90	1960.46 ± 132.26	**0.20** ± 0.06	**0.15** ± 0.03	<0.01 ± <0.01	20.64	9.22	8.12	<0.01
Syllable number	6.35 ± 0.19	2.00 ± 0.73	1.38 ± 0.30	0.33 ± 0.32	3.64 ± 0.33	**0.31** ± 0.12	**0.22** ± 0.05	0.05 ± 0.05	35.15	19.70	16.40	7.98
Phrase length	0.07 ± 0.01	<0.01 ± 0.01	0.02 ± <0.01	<0.01 ± 0.01	0.05 ± <0.01	0.02 ± 0.08	**0.21** ± 0.06	0.06 ± 0.07	34.81	5.26	15.88	8.72
Peak frequency	0.98 ± 0.03	0.04 ± 0.05	0.25 ± 0.03	<0.01 ± <0.01	0.71 ± 0.03	0.04 ± 0.05	**0.25** ± 0.03	<0.01 ± <0.01	31.66	6.51	15.92	<0.01
Maximum frequency	1.29 ± 0.10	1.25 ± 0.33	0.17 ± 0.06	<0.01 ± <0.01	0.49 ± 0.06	**0.96** ± 0.21	**0.13** ± 0.05	<0.01 ± <0.01	11.21	11.06	4.05	<0.01
Proportion unique syllables	0.02 ± <0.01	<0.01 ± <0.01	0.01 ± <0.01	<0.01 ± <0.01	0.01 ± <0.01	0.11 ± 0.06	**0.35** ± 0.04	0.08 ± 0.05	17.31	5.62	10.22	4.84

was no treatment effect on male body mass ($F_{1,25.7} = 0.62$, $P = 0.438$) but females from the control treatment were significantly heavier than those from the food-restricted group ($F_{1,41.2} = 8.59$, $P = 0.006$, $d = 0.74$). Male nestlings in the restricted group had significantly higher circulating corticosterone levels than controls at day 30 (mean corticosterone [pg/mL] ± SE: controls = 6521 ± 873; restricted = 8061 ± 966; $F_{1,70.6} = 4.24$, $P = 0.043$, $d = 0.24$), but although a similar trend was evident in females, it fell just short of statistical significance (mean corticosterone [pg/mL] ± SE: controls = 6323 ± 920; restricted = 8368 ± 939; $F_{1,84} = 3.66$, $P = 0.059$).

Discussion

In this study, we aimed to quantify the contributions of the rearing environment and genetics to male brain, body, and song traits, and to determine whether environmental challenges in the form of nutritional restriction affected the strength of these relationships. Specifically, we sought to test for significant G × E interactions in relation to stress treatment and found effects on brain mass and the proportion of the unique syllables in the song. These results suggest that different genotypes have greater trait expression under different regimes of nutritional availability. Theoretical work suggests that G × E can maintain additive genetic variation underlying a trait. However, these traits may only be under strong sexual selection if populations have stable environmental conditions, or have low dispersal (Greenfield and Rodriguez 2004; Kokko and Heubel 2008).

HVC is arguably the most important area of the oscine brain for the learning and production of song (DeVoogd et al. 1993; Yu and Margoliash 1996; Lovell et al. 2008), so one of the most striking results of this study is that it appears uniquely susceptible to the effects of environmental variation. Neither genetic origin nor a common rearing environment could explain a significant portion of the variation in HVC volume. Instead, virtually all of the variance was attributable to the error term, which includes all environmental factors, apart from common rearing environment, and any G × E effects that did not arise from the experimental treatment. The mild nutritional restriction induced in this study was designed not to impair neural development, but to ensure there was environmental variation between groups. This nutritional restriction had no detectable effect on HVC development, in contrast to the more extreme levels of stress used by previous studies (Nowicki et al. 2002; Buchanan et al. 2004; MacDonald et al. 2006), but in line with Gil et al. (2006) who used brood size manipulation to induce stress. The development of the HVC, and presumably the song traits it controls, were largely determined by environmental factors (potentially including more extreme levels of developmental stress).

Narrow-sense heritability (h^2) is an enduring measure of the portion of phenotypic variance ascribable to genetic control. It is of central importance to predicting the response of a trait to selection and allows traits to be compared with and across populations (Visscher et al. 2008), and its relevance continues to increase with advances in gene expression studies. Although estimates of h^2 are unique to the population in which they are measured, in practice they are often remarkably similar across populations and even between species (Visscher et al. 2008), so biologically meaningful estimates made in captive zebra finches have much to tell us about the way song, and its underlying neuroanatomy, respond to selection in other songbirds.

Heritability estimates were low for all brain variables and most song traits, with the exception of syllable number and the maximum frequency reached in song. Syllable number had a heritability of 0.31 and a coefficient of additive genetic variation (CV_A) of 19.7, comparable to the mean value of 16.8 that Pomiankowski and Moller (1995) found for secondary sexual traits in their meta-analysis across a range of species. CV_A standardizes the additive genetic variance of a trait with respect to the mean value of that trait, and represents a measure of evolutionary potential of the trait to selection (Charlesworth 1984; Houle 1992). Syllable number could thus be under selection as a signal of indirect (genetic) benefits offered by males. Syllable number is, indeed, associated with female preference in many species including the zebra finch (Catchpole and Slater 2008; Riebel 2009). The other song parameters we investigated have lower values of CV_A and would be unlikely to respond to selection.

In common with our results, Forstmeier et al. (2009) found generally low heritability for a number of male song traits in the zebra finch, although our estimate of the heritability of syllable number in the song phrase is roughly twice that reported by Forstmeier et al. (2009). Our estimates of the heritability of brain variables and body mass did not agree closely with those obtained by Airey et al. (2000a), who estimated that HVC volume and total brain mass both had moderate heritability (of 0.38 and 0.49, respectively), whereas RA volume was highly heritable ($h^2 = 0.72$). Sib–sib comparison indicated that RA volume, alone of the brain variables we addressed, was significantly heritable but, at 0.20, to a far lesser degree than indicated by Airey et al. (2000a). We found that neither HVC nor brain mass were significantly heritable. The low levels of heritability suggested by our results better reflect the fact that the song control nuclei of the avian brain are known to exhibit high levels of plasticity (Brainard and Doupe 2002; Brenowitz and Beecher 2005). The heritabilities calculated by Airey et al. (2000a) are certainly overestimated because they also include the effect of common rearing environment (song tutoring and provisioning), and were unable to investigate the possibility of any G × E interaction influencing brain development. Furthermore, our experimental treatment introduced greater levels of environmental variability than were present in Airey et al. (2000a) which will also have contributed to lower heritability estimates. The difference in heritability estimates for brain structure between this study and Airey et al. (2000a) demonstrates the stimulatory effect of song tutoring and other aspects of the rearing environment on neural development. Heritable factors accounted for 62–75% of the variation in body size in this study, approximately twice as much as found by Airey et al. (2000a). Maximum frequency is likely to be highly dependent on body size (Hardouin et al. 2007; Mager et al. 2007) and also appears highly heritable (h^2 estimated at 0.56 using father–son regression and 0.96 in sib–sib comparison). It should be noted that, although our sound recording protocol was designed to minimize variation in amplitude, it is possible that our measurements of maximum frequency could be affected to some degree by differences in amplitude, as measurements were taken from spectrograms rather than power spectra (Zollinger et al. 2012).

Common rearing environment explained between 13% and 35% of the variation in all five song variables we examined, reflecting the fact that birdsong is learned from a tutor (Marler 1990; Catchpole and Slater 2008). Interestingly, common rearing environment also explained a significant portion of the variance in RA volume, comparable to that explained by additive genetic effects. Thus, unrelated zebra finches that were reared together are more similar to one another in brain morphology than predicted by chance, demonstrating the direct influence of the song-learning environment on neural development as well as on song itself. Prior studies have shown that a common rearing environment predicted variation in HVC and the lateral nucleus magnocellularis anterioris (lMAN) volume as well as RA volume (Buchanan et al. 2004), whereas the complexity of tutor song was found to influence HVC but not RA structure in Eastern marsh wrens (*Cistothorus palustris*; Airey et al. 2000b). A number of previous studies have showed that disruptions to the song learning process (e.g., deafening, rearing in acoustic isolation or white noise) lead to abnormal development of song control nuclei including HVC, RA, and lMAN (reviewed in Kirn 2010). The influence of the learning environment on neural development thus appears to be a robust phenomenon. Three of the song parameters, syllable number, phrase length, and peak frequency, had a coefficient of variation attributable to common rearing environment (CV_{EC}) of 15.88–16.40, comparable to the CV_As of sexual signals discussed above, and have the potential to signal the quality of an individual's song tutor. This might indirectly signal genotypic quality in spite of the low heritability of song traits, because song is typically learned from the father (Miller 1979; Clayton 1987; Zann 1996).

There was a significant interaction between song tutor and nutritional treatment in determining the proportion of unique syllables in sons' songs, indicating that environmental conditions disrupt the link between tutor and song learning. If females

express a preference for song complexity, as has been shown in a number of species (Catchpole and Slater 2008), different environmental conditions will favor different genotypes and no one genotype is always superior. Theoretical work suggests that, under certain conditions, this provides a force to maintain additive genetic variation, even in the face of intense selection (Turelli and Barton 2004; Kokko and Heubel 2008). Phenotypic variation due to G × E effects, acting in concert with the condition-dependence of male ornaments, can lead to variation in male attractiveness, promoting sexual selection by female choice. If, however, dispersal mechanisms lead to females choosing between males from different environmental backgrounds this will "blur" the link between trait expression and male quality, eroding female choice (Kokko and Heubel 2008).

There was also an interaction between the experimental treatment and genotype, responsible for 40% of the observed variance in brain mass. Any link between brain mass and genotype can be disrupted by variation in the quality of the rearing environment, so traits linked to brain mass may be of little use in advertising genetic quality (Greenfield and Rodriguez 2004; Higginson and Reader 2009). Indeed, there is very little evidence that brain size is predictive of any specific cognitive trait (Healy and Rowe 2007; but see Kotrschal et al. 2013), in which case there may be no reason to expect any indicator of brain mass to be selected for. Several recent studies have suggested a link between song complexity and other cognitive traits in zebra finches (Boogert et al. 2008) and European starlings (*Sturnus vulgaris*; Farrell et al. 2012). This may reflect an underlying commonality in the way environmental challenges act on different parts of the brain (Buchanan et al. 2013) but, given our results, it seems likely that these learning abilities are specific and controlled by specialized brain nuclei that develop independently of overall brain mass.

Previous studies have suggested an inhibitory effect of male siblings on song development, in which male zebra finches with more male nest-mates or males in larger tutor-groups sang shorter songs consisting of fewer syllables (Tchernichovski and Nottebohm 1998; Gil et al. 2006), learned their tutors' songs less accurately (Tchernichovski and Nottebohm 1998), and even sang less to females in courtship (Ruploh et al. 2013). By contrast, we found no effect of either the number of male nest-mates or overall brood size on any song variable. A key difference between our study and previous work (Tchernichovski and Nottebohm 1998; Gil et al. 2006) may be that we had a narrower range of group sizes (1–3 male nestlings per nest in our study, compared to 1–5 in Tchernichovski and Nottebohm 1998 and 3–9 in Gil et al. 2006). No prior studies have directly tested for an effect of male nest-mates on the song control system, but Sartor and Ball (2005) have shown that social suppression of song in European starlings leads to reduced HVC volume. We found a weak positive relationship between the number of male nest-mates and HVC volume, the inverse of the expected relationship if male nest-mates inhibit singing behavior. Sockman et al. (2009) showed that European starlings exposed to playback of long songs developed larger RA than those exposed to short song, suggesting a stimulatory effect of listening to the song of others, which might explain the weak positive relationship between HVC and number of male nest-mates found here.

Our nutritional stress treatment had a significant adverse effect on the growth of female offspring but not that of males. Similar sex-specific effects of nutritional restriction on growth rates have been reported in song sparrows (Schmidt et al. 2012), and in hand-reared zebra finches (Martins 2004). In experiments on captive zebra finches, Kilner (1998) found female-biased nestling mortality when food availability was low; Arnold et al. (2007) found that females, but not males, reared under food restriction had shorter wings as adults; and De Kogel (1997) found that females from larger broods had a higher adult mortality rate, whereas brood size did not affect male mortality. Together these data strongly suggest that males and females make different trade-offs in the face of deleterious developmental environments. In our study, although the effect of nutritional stress on corticosterone levels was significant only for male nestlings, both males and females showed a similar trend in which circulating corticosterone levels were raised in chicks on a restricted diet. Although there appear to be sex-specific differences in the way nestlings respond to developmental stress, the effect of stress on circulating hormone levels appears similar in males and females.

This study has provided estimates for the heritability of the volume of two of the most important neural loci for the learning and production of oscine song. For the first time we have been able to separate the effects of additive genetic variation from those of a common rearing environment and to look for an influence of G × E interactions. Our results indicate that although RA volume is moderately heritable, HVC volume has extremely low heritability, suggesting that environmental factors play an extremely important role in regulating its development and function. This study confirms that most aspects of song structure are unlikely to be under selection to reflect heritable quality. Our study demonstrates the fundamental importance of environmental conditions not only for vocal learning in songbirds, but also for the potential for selection to act on the expression of singing behavior and neural development.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S3. Results of father-son regressions in which the model was constrained to retain brood size and the number of male nest-mates as covariates.