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ENVIRONMENTAL STUDIES

Traffic noise disrupts vocal development and suppresses immune function

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Noise pollution has been linked to learning and language deficits in children, but the causal mechanisms connecting noise to cognitive deficiencies remain unclear because experimental models are lacking. Here, we investigated the effects of noise on birdsong learning, the primary animal model for vocal learning and speech development in humans. We found that traffic noise exposure retarded vocal development and led to learning inaccuracies. In addition, noise suppressed immune function during the sensitive learning period, indicating that it is a potent stressor for birds, which is likely to compromise their cognitive functions. Our results provide important insights into the consequences of noise pollution and pave the way for future studies using birdsong as an experimental model for the investigation of noise-induced learning impairments.

INTRODUCTION

Noise pollution has been classified as one of the main environmental threats to public health (*1*). In the European Union alone, more than 100 million people are affected by hazardous noise levels from vehicle and aircraft traffic, and this number is projected to increase because of future urban growth and an increased demand for mobility (*2*). Although traffic noise levels are usually too low to cause physical damage to the ear, chronic noise exposure at equivalent levels of 55- to 60-dB(A) sound pressure level (SPL) can already cause severe nonauditory health effects such as a considerable increase in the risk of cardiovascular and metabolic diseases that may lead to premature mortality (*1*, *3*). In addition, noise exposure has been linked to impaired cognitive function, such as learning and language deficits in children (*3*–*5*). In both adults and children, chronic noise can lead to an increase in the levels of biomarkers for stress, such as catecholamines and glucocorticoids (*3*, *6*). Two pathways have been accounted for this response: emotional stress reactions due to perceived discomfort and nonconscious physiological stress from direct interactions between the central auditory system and other brain regions (*3*). However, the causal mechanisms linking chronic noise exposure, stress, and learning impairments in children have not been clearly identified.

Birdsong learning is a paradigmatic model system for vocal learning and speech development in humans (*7*, *8*) and thus can provide valuable clues to the understanding of the observed learning and language deficits in children exposed to chronic noise. Like human children, songbirds must learn their vocalizations (mainly songs) from adult tutors during a sensitive period in their ontogeny (*7*, *9*). Young zebra finch (*Taeniopygia guttata*) males develop individually distinct songs by imitating adult males (tutors) (*10*), a process of sensorimotor learning spanning several months (*11*). Their songs consist of complex sounds (syllables) that are repeated in a fixed order (motifs) (*12*). The sensitive period for song memorization in zebra finches starts after 25 days post hatch (dph) (*13*). Then, during the sensorimotor period between 35 and 90 dph, tutees develop their song by gradually matching the phonetic morphology of their song syllables to the memorized tutor template (*14*). At 90 dph, zebra finch song usually crystallizes, i.e., phonetic syllable changes have reached a plateau, and the song remains largely unchanged afterward (*11*).

Earlier studies on vocal learning in zebra finches and other songbirds found that exposure to very high levels of white noise prevented normal song learning and behavior and was often used as a reversible alternative to deafening birds in experimental studies. When noise levels are high enough to mask all other acoustic input, young birds fail to learn their species-typical songs, producing instead atypical songs that resemble songs of deafened birds (*15*–*18*). At extreme levels, noise exposure can result in temporary or permanent hearing loss in birds, but noise levels encountered in cities are typically not high enough to cause temporary threshold shifts in birds (*19*). Impairments of cognitive learning in children, however, can already occur at low levels of short-term noise exposure of 48-dB(A) SPL peak amplitude (*20*). Moreover, chronic exposure to artificial noise at 40- to 80-dB(A) SPL has been shown to affect the accuracy of song copying in zebra finches (*21*). Still, the effects of realistic anthropogenic noise, such as birds and humans are exposed to in urban areas, are unknown, and in particular, the impacts on song development remain unexplored.

We addressed two questions: First, we explored whether realistic traffic noise disrupts the timing of vocal development and impairs learning in zebra finches. Second, to elucidate the mechanism underlying potential noise-related differences, we investigated whether traffic noise is a stressor to the birds and whether biomarkers for stress are related to learning success. Our data comprise trajectories of vocal development and the song learning success of zebra finches that were raised and song tutored in sound-shielded boxes, in combination with measures of their plasma corticosterone levels and stress-related changes in immune function. Noise-treated birds were exposed to recordings of urban traffic broadcast at realistic sound levels during the sensorimotor period and early adulthood (18 to 100 dph).

RESULTS AND DISCUSSION

We found that the noise-exposed birds had delayed song development compared to the controls. In controls, the biggest developmental

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progress was observed between 60 and 90 dph, after which their songs crystallized and syllable variation remained stable, whereas in the noise-treated birds, the ontogenetic changes were more gradual and their element stereotypy was lower between 60 and 105 dph [all $P(\beta) > 0.98$; Fig. 1A]. At day 120, the noise birds had caught up and reached stereotypy values in their crystallized song similar to those of the control birds [$P(\beta) = 0.84$; variance of random effect bird ID, 3.338 ± 1.827; residual variance, 12.918 ± 3.594; $N = 7019$ observations of 26 birds; marginal $R^2 = 0.630$; conditional $R^2 = 0.706$]. Song similarity between the tutor song and the crystallized adult song of the tutees at 120 dph (Fig. 1B) was lower in noise-treated birds, indicating that they developed poorer copies of the model song than control birds ($N_{\text{control}} = 12$, $N_{\text{noise}} = 17$, $P(\beta) = 0.972$; adjusted $R^2 = 0.10$; Fig. 1C).

When analyzing the corticosterone levels of the birds in our experiment, we found no statistically meaningful difference between noise-treated birds and controls on any of the sampled days (fig. S1A). The same result was also obtained with a second dataset including male and female birds (fig. S1B). Tutees with a lower learning success had slightly higher corticosterone concentrations than males with a higher learning success, but the 95% credible intervals of the slope included zero (slope, −3.605 [−8.174 to 0.917]), suggesting no systematic influence of corticosterone on learning success. However, we found a marked effect of the noise treatment on the birds' immune system: While the noise-exposed birds showed a similarly strong response to PHA (phytohemagglutinin) injection as the controls did after 24 hours [$P(\beta) = 0.91$, adjusted $R^2 = -0.02$; Fig. 2A), their immune function was suppressed after 48 hours [$P(\beta) = 0.99$, adjusted $R^2 = 0.26$; Fig. 2B]. This pattern shows that noise-exposed birds were unable to maintain a normal immune response, indicating a pronounced chronic stress effect (*22*). In general, learning success varied with the 48-hour PHA response (slope, 0.419 [0.038 to 0.802]; Fig. 2C), i.e., tutees with weaker immune responses developed poorer song imitations than males with a stronger immune response.

Noise-induced stress notwithstanding juveniles in the noise treatment group sang as much as the control birds during the sensorimotor phase of vocal learning (fig. S1C). This indicates that the delay in development and the lower learning success was not due to a lack of vocal practice. Moreover, singing activity at 74 dph generally did not predict song learning success (slope, −0.003 [−0.009 to 0.002]), which supports the notion that the vocal activity during the sensorimotor period is not a form of vocal practice determining the accuracy of song imitation (*23*, *24*).

Our findings also suggest that traffic noise pollution has the potential to affect the cultural evolution of birdsong (*25*, *26*) because noise-induced copying errors are likely to accumulate as song passes from one bird to another. Increased error rates would thus lead to less conformity (*27*) in noise-exposed populations until genetic constraints prevent further variation (*28*, *29*). Ultimately, the learning inaccuracies and the delayed vocal development can contribute to diverging lines of vocal cultures in noise-polluted habitats (*30*). Potential fitness consequences of altered vocal traditions, as well as other consequences of the observed learning impairments, remain to be investigated.

To summarize, we show that experimental traffic noise exposure delayed song development in zebra finches by ca. 30% and led to a significantly lower learning success. This finding parallels noise-related

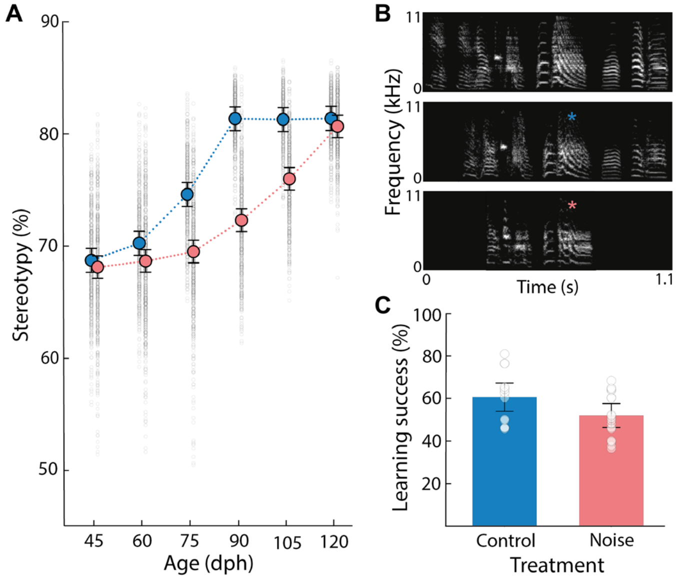

Fig. 1. Traffic noise affects song learning and vocal development. (**A**) Ontogenetic trajectory of song development measured as intraindividual syllable variation. Stereotypy quantifies the matching of spectrotemporal parameters between different renditions of the same syllable type; as the song development progresses, accuracy increases, i.e., syllable variation decreases and songs become more stereotyped. Red, noise-exposed birds; blue, control birds. Symbols and error bars represent posterior Bayesian mean estimates with their 95% credible intervals. Open dots denote individual data points. Differences between groups can be considered statistically meaningful if the 95% credible intervals of one group do not overlap with the mean estimate of another group. (**B**) Tutor song motif (top) and adult song motifs (120 dph) of a good learner (control tutee with 81% similarity to the tutor song; middle) and a poor learner (noise-exposed tutee with 44% similarity to the tutor song; bottom). The syllables with the highest entropy variance, which were used for the tracking of the developmental trajectory (A), are marked with an asterisk. (**C**) Effect of noise treatment on learning success [similarity between crystallized (120 dph) tutee song and tutor song]. Posterior means and 95% credible intervals; open dots denote individual data points.

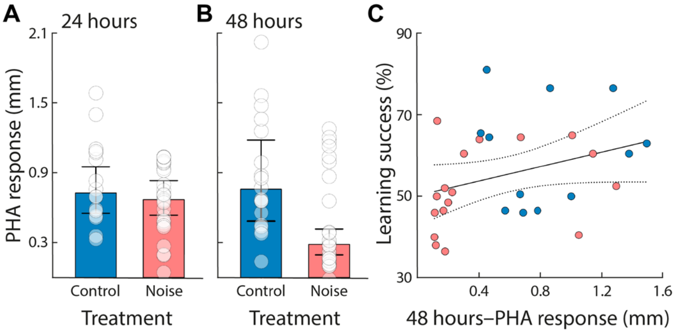

Fig. 2. Traffic noise exposure suppresses immune function in juvenile zebra finches, and immune function predicts learning success. (**A**) Posterior mean and 95% credible intervals of PHA skin response (change in patagium thickness) after 24 hours. Open dots represent individual data points. (**B**) PHA skin response after 48 hours. (**C**) Correlation between PHA response after 48 hours and song learning success [similarity between crystallized (120 dph) tutee song and tutor song]. Red, noise-exposed birds; blue, control birds. Note that the sample sizes in (A) and (B) are larger because PHA responses were also measured in additional birds for which song was not recorded (fig. S1B).

impairments of language competence and learning in human children (*4*, *31*). Our results indicate that young songbirds, just like human children, are particularly vulnerable to the effects of noise because of its potential to interfere with learning at a critical developmental stage.

Auditory noise effects may reduce the audibility of model songs (*32*) and affect auditory feedback during song development (*33*). However, the fluctuating noise levels in our experiment were not high enough to mask the model songs or to impair the tutees hearing (*32*). Rather, we found evidence for indirect noise effects in terms of a stress response. Juvenile birds are particularly sensitive to noise-induced stress (*34*), and here, we show that a noise stressor can suppress the immune function of young birds during the sensitive learning period. In addition to noise-induced stress, vocal learning and development in juveniles can also be directly affected through noise effects on auditory brain development, which manifest themselves in poorer acoustic processing and delayed neural maturation (*35*, *36*). Juvenile zebra finches that were exposed to chronic artificial noise developed smaller HVC nuclei (*21*), a brain region necessary for both song learning and production (*37*). In human children, similar noise effects on auditory brain development may lead, e.g., to linguistic deficiencies and impairments of speech understanding (*38*).

Although noise-related learning deficiencies remain a complex phenomenon, our approach makes it possible to assess the adverse effects of noise pollution on auditory memory and behavioral motor skills in an animal system. It remains to be shown whether noise pollution also affects memory and learning in other modalities, such as vision or chemoperception. The quantitative methods established in the study of birdsong learning allow measuring motor learning success and tracking the entire ontogenetic trajectory of vocal development, opening up new avenues using birdsong as an experimental model to investigate noise-related cognitive and ontogenetic impairments, especially learning deficiencies and retardation of speech development.

MATERIALS AND METHODS

Experimental setup and procedure

We used 36 male zebra finches that were bred from birds from the colonies at the Max Planck Institute for Ornithology in Seewiesen, Germany. The young were raised and tutored by playback in cages in sound-shielded boxes to control their auditory input and to track their vocal ontogeny. Details of the husbandry, the sound boxes, and the experimental procedures including details of the noise recordings have been described previously (*39*). Briefly, the boxes were equipped with two loudspeakers mounted 149 cm apart, one in each of the small sides of the box. Lighting was set on a 12-hour light/12-hour dark cycle (lights on from 0700 to 1900 hours). Eight days after hatching, all young were genetically sexed and then swapped between breeding pairs so that all nests contained two male chicks and one or two female chicks. On 17 dph, i.e., before young zebra finches start memorizing model songs (*13*), the male partner of each pair was removed, and from 18 to 100 dph, the young birds were tutored with a song playback six times per day (at 0800, 0900, 1100, 1500, 1600, and 1800 hours). Each playback session consisted of 42 motifs of a specific zebra finch song type that had yielded high learning success in previous experiments (Fig. 1B), broadcast with a peak amplitude of 75 dB(A) (re. 20 μPa) at the position of the perches in the cage [while the A-weighting is modeled on human sound perception, the similarity in audibility curves makes A-weighted SPLs also a good proxy for bird hearing (*19*, *40*)]. The tutoring files consisted of recordings of three natural singing bouts [including the typical soft introductory notes (*12*)] that were played back several times in random order in each session. In the noise treatment, birds were also exposed from 18 to 120 dph to traffic noise that had been recorded in bird habitats close to busy roads in the city of Munich, Germany. During the day, the peak noise level ranged between 60- and 80-dB(A) SPL (at the position of the perches) and during nighttime between 50- and 70-dB(A) SPL, mimicking typical values of urban traffic noise (*2*). Comparisons of power spectra showed that our playback system reproduced the traffic noises with high fidelity: At any frequency in the band between 0.1 and 10.0 kHz [which covers the entire hearing range of zebra finches (*41*)], the noise playback deviated less than 3 dB when broadcast in the sound boxes compared to the original recordings from the urban areas. While the noise was played through both loudspeakers, simulating a homogeneous noise field, the tutor songs were only broadcast from one loudspeaker to simulate one singing tutor male. In the control treatment, only the tutor songs were played back and no traffic noise. Sound levels inside silent sound boxes (i.e., when no playback was broadcast and excluding bird noises) ranged between 31- and 33-dB(A) SPL and were mainly due to the ventilation system.

Song recording and analyses

The singing activity of each male was automatically detected and saved (sampling frequency, 44.1 kHz; accuracy, 16 bits) with the Sound Analysis Pro (SAP) 2011 software (*42*). On recording days (see below), each cage was divided with a metal grid into two equal compartments to separate the two males. Each male's vocal output was then recorded by one of two microphones that were suspended above the perch in the respective compartments (the recordings could be assigned unequivocally to each male because of the amplitude differences between the two recording channels, but in most cases, the song types of the two males in each box were sufficiently different to discriminate them anyway).

Vocal development

The trajectory of song development was quantified by measuring the intraindividual syllable variation (i.e., stereotypy) at 45, 60, 75, 90, 105, and 120 dph (±1 day in some cases due to differences in hatching dates or for logistic reasons). As vocal development in zebra finches progresses, their song syllables become increasingly stereotyped until song crystallization. Stereotypy was computed as described previously (*42*) using the accuracy measure of SAP, which indicates how well the spectrotemporal parameters of two sounds are matched. For 14 noise-treated males and 12 control males, we selected their most complex syllable type, which was determined as the syllable with the highest entropy variance (*42*) at 45 dph (measured across 10 renditions for each bird; Fig. 1B), and compared 10 renditions of this syllable from each developmental stage. This resulted in 45 pairwise accuracy measurements for each male and developmental stage. Before the measurements, the amplitude of the selected syllables was normalized to 90% using the software Avisoft-SASLab Pro. 5.2.08 (Avisoft Bioacoustics, Berlin, Germany). During the plastic song stage, zebra finches undergo a typical diurnal pattern of song changes, in which the quality of song imitations increases during the day and then regresses overnight (*18*, *43*). To account for this, we only considered song recordings from between

1700 and 1800 hours for the tracing of the developmental trajectory. During this period of time, the noise was switched off.

Learning success

The learning success of the birds was computed as described previously (*10*, *42*, *43*) using the similarity score of SAP. Briefly, this procedure quantifies the similarity between songs on the basis of several acoustic parameters: frequency modulation, amplitude modulation, pitch, goodness of pitch (an estimate of harmonic pitch periodicity), and Wiener entropy (a measure of the width and uniformity of the power spectrum). Using the batch module of SAP, we computed 10 comparisons between crystallized tutee song motifs (120 dph) and the model song for 17 noise-treated males and 12 control males and then used the median similarity score of each bird for further analyses.

Corticosterone and PHA measurements

To investigate whether the noise exposure elicited a physiological stress response in the birds, we measured two parameters: plasma corticosterone concentrations and immunocompetence. Plasma corticosterone was measured at 15, 45, 75, and 120 dph. For this purpose, blood samples were collected with heparinized capillary tubes (1.4 × 75 mm) from the brachial vein, transferred into Eppendorf tubes, and centrifuged at 5000 rpm for 10 min to separate the plasma. Plasma corticosterone concentrations were determined by radioimmunoassay as reported previously (*44*). Corticosterone antibodies were obtained from Esoterix Endocrinology, Calabasas Hills, CA. Extraction efficiency (as calculated from tracer amounts of tritiated hormone added to each sample before extraction) was 80.0 ± 5.4% ($N = 183$) for ^{3}H-corticosterone (PerkinElmer, NET 399). The average limit of detection was 35- and 42-pg corticosterone/ml for the two corticosterone assays that we ran. The intra-assay variation for corticosterone standards was 3.3 and 5.0% and 17.0 and 1.7% for extracted chicken pool plasma. Interassay variation of unextracted standard corticosterone was 11.8% and was 19.4% for extracted chicken pool plasma.

As a second measure of stress, we quantified the birds' immune responses at 75 dph. Chronic stress suppresses immune functions in birds and other vertebrates (*22*); thus, an impaired immune response can be indicative of a stressor. To assess potential stress-related changes in immune function, we conducted a PHA skin test. This test entails an intradermal injection of PHA in the patagium (wing web) of the birds. PHA is a T cell mitogen commonly used to assess cell-mediated immunity in birds (*45*, *46*). The PHA injection stimulates a local infection triggering recruitment of immune cells to the injection site and causing a local inflammation of the skin. For each PHA injection, we used reagent-grade PHA (Remel Inc., HA15/30852701 HA15) dissolved in sterile phosphate-buffered saline (PBS) in a treatment dosage of 0.15-mg PHA per 30 μl of the buffer. Before injection, the patagia of each bird were cleaned with alcohol, and then, the PHA solution was injected into the right patagium. Then, as a control, the left patagium of the same bird was injected with 30 μl of PBS. A pressure-sensitive micrometer (Dyer 304-196) was used to measure patagium thickness immediately before the injection and again 24 and 48 hours after injection. Each measure was taken four times, and then, average values per bird were used to calculate the PHA response as the change in thickness of the PHA-treated patagium (post-injection thickness minus pre-injection thickness) minus the change in thickness of the control patagium.

Singing activity

If traffic noise is a stressor, then it may lead to changes in activity budgets; hence, noise-exposed tutees may sing less and thus have less vocal practice during song development. Therefore, we quantified the singing activity at 74 dph of 14 noise-treated males and 12 control males. The duration of all song bouts (separated by pauses >0.5 s) was measured in waveforms with Avisoft-SASLab Pro to yield an overall score of singing activity for the entire day.

Statistical analyses

Statistical analyses were performed with R version 3.6.2 "Dark and Stormy Night" (R Development Core Team, Vienna, Austria, 2019) using a Bayesian statistical approach with the R packages "arm" (*47*) and "lme4" (*48*). In contrast to a frequentist statistical approach, Bayesian statistics allow the likelihood of a true difference between groups to be estimated. Song accuracy was analyzed using a linear mixed model (function "lmer" of lme4) with treatment and dph as fixed effects and bird identity as a random effect. All other parameters were analyzed using linear models (function "lm" in lme4). We assessed model residuals with diagnostic plots (qq plots of residuals, fitted values versus residuals) for homogeneity of variance, violation of normality assumptions, or other departures from model assumptions and model fit. For inferences from the models, we calculated Bayesian parameter estimates and their 95% credible intervals [based on 10,000 simulations of the function bsim of the R package arm (*47*) with an uninformed prior distribution (*49*)]. In frequentist statistics, the statistical test provides a P value describing the probability that the null hypothesis is true given the data. Bayesian statistics does not provide such P values. Instead, Bayesian statistics directly tests for meaningful differences between groups, which can be assessed by comparing the ranges of the 95% credible intervals between these groups. The posterior mean and 95% credible interval provide an estimate for the respective group mean with a probability of 0.95 (*49*). If the credible interval of one group does not overlap with the mean estimate of another group, then the groups can be assumed to differ from each other. We also provide the posterior probability $P(\beta)$ of the likelihood that the parameter estimates differ from each other, with values of $P(\beta)$ close to one indicating statistically meaningful differences between groups. In addition, we mention how much of the variance is explained by our models (goodness of fit) by reporting R^2 values for linear models and the respective marginal and conditional R^2 values for mixed models as described previously (*50*). The marginal R^2 value represents the variation explained by the fixed effects of a mixed model, whereas the conditional R^2 value reflects the combined variation explained by fixed and random effects. If not indicated otherwise, data are presented as individual data points in combination with Bayesian posterior means and their respective 95% credible intervals (reported in squared brackets).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/20/eabe2405/DC1

View/request a protocol for this paper from *Bio-protocol*.

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Traffic noise disrupts vocal development and suppresses immune function

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