Ambient noise induces independent shifts in call frequency and amplitude within the Lombard effect in echolocating bats

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The Lombard effect, an involuntary rise in call amplitude in response to masking ambient noise, represents one of the most efficient mechanisms to optimize signal-to-noise ratio. The Lombard effect occurs in birds and mammals, including humans, and is often associated with several other vocal changes, such as call frequency and duration. Most studies, however, have focused on noise-dependent changes in call amplitude. It is therefore still largely unknown how the adaptive changes in call amplitude relate to associated vocal changes such as frequency shifts, how the underlying mechanisms are linked, and if auditory feedback from the changing vocal output is needed. Here, we examined the Lombard effect and the associated changes in call frequency in a highly vocal mammal, echolocating horseshoe bats. We analyzed how bandpass-filtered noise (BFN; bandwidth 20 kHz) affected their echolocation behavior when BFN was centered on different frequencies within their hearing range. Call amplitudes increased only when BFN was centered on the dominant frequency component of the bats' calls. In contrast, call frequencies increased for all but one BFN center frequency tested. Both amplitude and frequency rises were extremely fast and occurred in the first call uttered after noise onset, suggesting that no auditory feedback was required. The different effects that varying the BFN center frequency had on amplitude and frequency rises indicate different neural circuits and/or mechanisms underlying these changes.

acoustic communication | audiovocal integration | Lombard speech | signal masking | vocalization

Any transmission of signals between sender and receiver faces the challenge of being subjected to masking by noise. For acoustic signals, for example, animals have evolved several strategies that aid in increasing the signal-to-noise ratio, thus facilitating signal transmission. One of the most efficient mechanisms is the so-called Lombard effect, i.e., the involuntary rise in call amplitude in response to masking ambient noise (1). This effect was first described in human communication a century ago (2) and has since been found in several species of birds (3–11) and various mammals (12–17), including bats (18). In human speech, several vocal changes, such as a rise in fundamental frequency (19) or lengthening in word duration (20), are often accompanied with the Lombard effect; combined, these changes are referred to as Lombard speech (21). Vocal changes associated with the Lombard effect have rarely been analyzed in animal species. So far, noise-dependent changes in call frequency have been observed in birds (8, 11, 22), and changes in call duration in birds and monkeys (8, 12).

Most animal studies, however, have focused on noise-dependent changes in call amplitude and do not examine other possible vocal changes (2–5, 7, 9, 10, 15–17). It is therefore still largely unknown how the adaptive changes in call amplitude relate to frequency shifts or call elongation and how the underlying mechanisms are linked. It is also unknown if auditory feedback

from the changing vocal output is needed to drive the Lombard effect in general.

Over the past decades, studies on the Lombard effect have proven relevant across a diverse range of scientific fields (21, 23); in addition to being a useful and important tool for diagnosing hearing loss, it is generally valuable for studies on vocal disorders and speech production. The Lombard effect has been used as a therapeutic tool to improve speech intelligibility in patients suffering from Parkinson's disease, and is also relevant to the study of phonetics and linguistics as well as in the context of animal behavior and the evolution of vocal plasticity (21). Therefore, studying the Lombard effect, the associated vocal changes, and their interaction between each other may lead to a better understanding of both the phenomenology and the underlying neurobiological control mechanisms (23).

Echolocating bats are an excellent animal model to study the effects of masking ambient noise on vocal behavior; they are highly vocal and constantly adjust their echolocation pulses to optimize signal detection by monitoring the returning echo signals, which represent the feedback from their own voice (24, 25). In addition, the bats' large hearing range of some 100 kHz (26) (Fig. 1A) allows one to determine the role of how masking of different frequency bands affects their echolocation behavior.

In the present study, we examined the Lombard effect and the associated vocal changes in call frequency by analyzing how bandpass-filtered noise (BFN; bandwidth 20 kHz) affected the echolocation behavior of greater horseshoe bats, *Rhinolophus ferrumequinum*, when the noise was centered at different frequencies within the bat's hearing range.

Results

We recorded echolocation pulses from three horseshoe bats emitted at rest (total number of calls: 83,073) in response to various masking conditions (Fig. 1B; see *Materials and Methods* for further details) and examined changes in such call parameters as call frequency and amplitude. Fig. 1C shows a representative example of a call sequence and illustrates the spectrotemporal composition of typical horseshoe bat echolocation calls emitted spontaneously and without acoustic stimulation. Horseshoe bats emit echolocation calls that are characterized by a long constant frequency (CF) component, and the CF frequency emitted while the bat is perched (at rest) is called the resting frequency (RF),
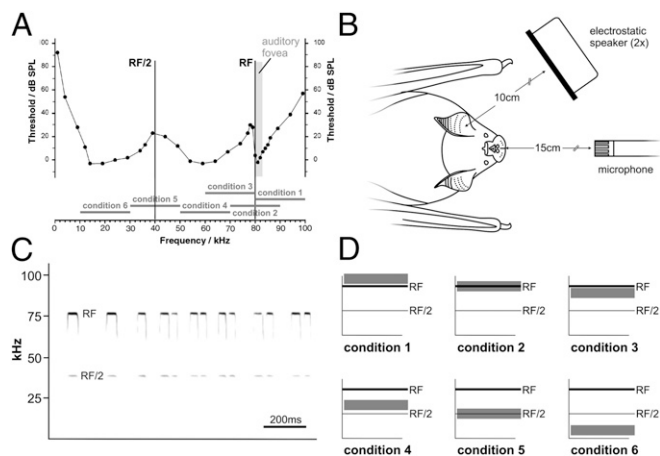
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Fig. 1. Behavioral setup and design of stimulus presentation. (*A*) Audiogram of horseshoe bats (modified from ref. 26) and its relationship with the frequency ranges covered by BFN (*D*). (*B*) Behavioral setup. A microphone was placed 15 cm in front of a stationary horseshoe bat. Acoustic stimuli were presented via two speakers (one for BFN, one for call echo), both placed 10 cm next to the bat's left ear. (*C*) Spectrograms of horseshoe bat echolocation calls showing the characteristic CF and FM components. A brief frequency component (FM) at the beginning (initial FM) and end (terminal FM) surround a long (>40 ms) constant frequency (CF) component, typically between 65 and 80 kHz in greater horseshoe bats. (*D*) Frequency ranges covered by BFN (horizontal bold lines; conditions 1–6). RF, resting frequency; RF/2, fundamental resting frequency. Condition 1: BFN extended 20 kHz above RF +500 Hz; condition 2: BFN centered on RF; condition 3: BFN extends 20 kHz below RF −500 Hz; condition 4: BFN centered 20 kHz below RF; condition 5: BFN centered on RF/2; condition 6: BFN presented between 10 and 30 kHz.

which normally represents the second harmonic of the calls. The pulses usually terminate with a brief and rapid drop in frequency and sometimes also contain an initial brief frequency rise, each extending 10–15 kHz below RF (24, 25). These long, narrow-band echolocation signals enable the bats to use echo cues caused by the wing beats of the flying insects upon which they prey. Fluttering insects cause frequency modulations in the returning echoes, which contain the information necessary for the bat to detect and even recognize its prey (27, 28).

To test the effects of masking on echolocation, we centered 20-kHz BFN stimuli on different frequencies, such as the bat's RF, the fundamental component of its RF (RF/2), as well as frequencies above and below RF (Fig. 1*D*). The frequency ranges tested covered virtually the entire hearing range of greater horseshoe bats (Fig. 1*A*). RFs of the three bats emitted without masking were very stable at 69.9 ± 0.05 kHz, 74.7 ± 0.05 kHz, and 76.2 ± 0.04 kHz, respectively, averaged over all experimental sessions and extending over a time period of 78 d.

Effects of BFN on Call Amplitude. Call amplitudes changed significantly when BFN was added to the playback signal for conditions 1, 2, and 4 averaged across all three bats ($P < 0.001$, df = 6, $\chi^2 = 941.6$, Kruskal–Wallis with post hoc bootstrap analysis for medians; Fig. 2*A*). Based on the population effect size (*Materials and Methods*), however, most of these changes did not represent large effects (−0.1 dB for condition 1 and 0.7 dB for condition 4; Table S1) and call amplitudes did not differ from the amplitudes during the "no noise" condition (threshold: 1.2 dB).

The largest increase in call amplitude was 2.2 dB and occurred only when noise was centered on RF (condition 2); it was significantly different from the results found for all other conditions, including 1 and 4. Interindividual differences for effects of BFN on call amplitude were found to be very small (median difference: 0.7 dB between all bats in all six conditions), and each of the three bats

exhibited significant differences in call amplitude when BFN was added to the playback signal [$P < 0.001$ for each bat, df = 6, $\chi^2 = 480.7$ (bat 1), 187.1 (bat 2), 772.6 (bat 3), Kruskal–Wallis with post hoc bootstrap analysis for medians; Fig. S1*A*)]. In each of the three bats, these shifts in call amplitude were significantly higher when BFN was centered on RF (condition 2) than during all other conditions (medians for condition 2 between 1.9 and 2.4 dB; Fig. S1*A*).

We then further analyzed the effects that masking with BFN centered on RF (condition 2) had on the bats' echolocation performance. First, we varied the intensity of BFN while keeping playback intensities constant, and then kept BFN intensities constant but varied the playback intensities. In the first scenario, we presented BFNs at 80, 90, and 100 dB sound pressure level (SPL) while maintaining the playback attenuation constant at 30 dB (Fig. 2*B₁*). We found that the call amplitudes increased significantly with increasing BFN intensities: a BFN of 80 dB SPL resulted in an average increase in call amplitude of 0.5 dB, which is 1.7 dB less than the average amplitude increase for a BFN at 100 dB SPL ($P < 0001$, df = 2, $\chi^2 = 234.2$, Kruskal–Wallis with post hoc bootstrap analysis for medians; Table S2). In the second scenario, playback attenuations of 10, 20, and 30 dB relative to call intensity (for constant noise amplitudes of 100 dB SPL) yielded average rises in call amplitudes of 1.3, 0.7, and 2.2 dB, respectively, which were all significantly different from one another ($P < 0001$, df = 2, $\chi^2 = 250.2$, Kruskal–Wallis with post hoc bootstrap analysis for medians; Fig. 2*B₂*).
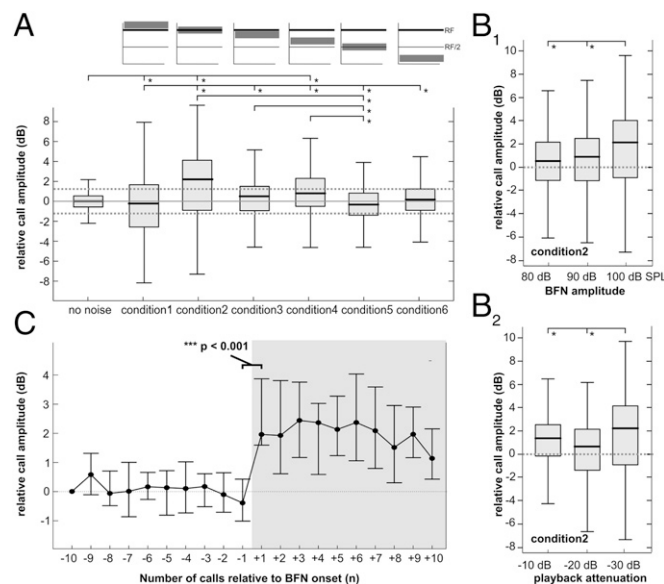

Fig. 2. Changes in call amplitude in response to BFN presentation. (*A*) Shifts in call amplitude in response to different BFN conditions (BFN amplitude: 100 dB SPL; playback attenuation: 30 dB) compared with the control (no BFN); data averaged for all three bats. Medians: horizontal lines inside boxes; first and third quartiles: upper and lower margins of boxes, respectively; 5% and 95% quantile: small horizontal bars above and below boxes, respectively. Dotted lines indicate the thresholds of the population effect size (±1.2 dB). (*B*) Effects of different BFN intensities (*B₁*) and playback attenuations (*B₂*) on call amplitude in condition 2. (*B₁*) Shift in call amplitude is slightly lower for weaker noise amplitudes (playback attenuation constant at 30 dB). (*B₂*) Shift in call amplitude is slightly higher for the lowest playback attenuation (30 dB). (*C*) Changes of call amplitude of the last 10 calls prior and the first 10 calls just after BFN presentation relative to the call amplitude of the 10th call before noise onset. Data averaged for all three bats and noise conditions 1–6 (36 trials; medians with first and third quartile). First calls uttered after noise onset show a significant increase in call amplitude (Kruskal–Wallis with post hoc signed-rank test).

Interestingly, call amplitudes increased extremely quickly after the onset of BFN. When we analyzed the time courses of the amplitude rises, we found that they already reached their maximum level for the very first call emitted after BFN onset ($P < 0.001$, df = 19, $\chi^2 = 156.9$, Kruskal–Wallis test with post hoc sign rank test; Fig. 2C). This finding was not related to any random amplitude fluctuations: Call amplitudes remained very stable for the last 10 calls before BFN onset, and the first 10 calls after the end of BFN, showing no significant differences between each other [$P > 0.05$, df = 9, $\chi^2 = 8.23$ (before) 9.03 (after) Kruskal–Wallis test].

Effects of BFN on Call Frequency. Whereas BFN yielded rises in call amplitudes only in stimulus condition 2, all six noise conditions resulted in significant increases in call frequencies averaged for all three bats ($P < 0.0001$, df = 6, $\chi^2 = 3546.6$, Kruskal–Wallis test, post hoc bootstrap analysis for medians; Fig. 3B), especially when BFN covered frequency ranges anywhere below RF. Fig. 3A shows a shift in response to BFN for condition 5, for which we found the largest rise, increasing call frequencies by an average of 269 Hz (Fig. 3B; Table S1). Even condition 2, in which only half of the BFN was presented below RF, call frequencies increased by 122 Hz on average for all three bats. BFN presented above RF (condition 1) showed the smallest yet still significant frequency increase ($P < 0.05$ for all BFN conditions, post hoc bootstrap analysis for medians) with a median rise of 24 Hz, which was significantly smaller than the other conditions ($P < 0.05$ for all BFN conditions, post hoc bootstrap analysis for medians). Based on the population effect size (*Materials and Methods*; threshold: 60 Hz), shifts in call frequency for condition 1 were determined not to differ from the "no noise" condition.

The effects of BFN on call frequency produced similar results in all three bats tested [$P < 0.001$ for each bat, df = 6, $\chi^2 = 814.3$ (bat 1), 981.2 (bat 2), 3281.0 (bat 3), Kruskal–Wallis with post hoc bootstrap analysis for medians; maximum median values between 122 and 341 Hz; Fig. S1B]. Though conditions 2–6 yielded significant increases in call frequency in each of the three bats, large interindividual differences were only found when BFN was centered 10.5 kHz above the bat's RF (condition 1). Though one bat
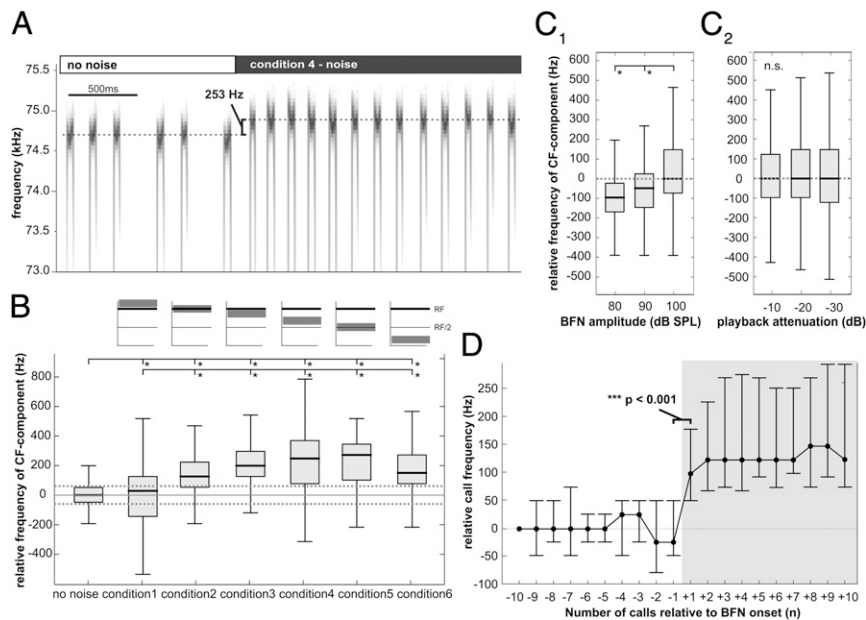
(bat 3) did not significantly change its call frequency, the other two bats either significantly increased (bat 2) or decreased their call frequencies (bat 1; Fig. S1B).

Similar to our analysis of BFN effects on call amplitudes (Fig. 2B), we also tested two additional scenarios in which we presented different BFN amplitudes while maintaining the playback attenuation constant (Fig. 3C₁) or changed the playback amplitudes while presenting BFN at a constant amplitude (Fig. 3C₂). The results presented in Fig. 3C represent averages for all three bats and all six stimulus conditions. We found that BFN increased call frequencies only in the first scenario when we presented BFN amplitudes of 80, 90, or 100 dB SPL, and maintained the playback amplitude constant at an attenuation of 30 dB. BFN of 80 dB SPL resulted in a frequency shift that was on average 98 Hz smaller than noise presented at 100 dB SPL (Fig. 3C₁; $P < 0001$, df = 2, $\chi^2 = 816.0$, Kruskal–Wallis-test with post hoc bootstrap analysis for medians; Table S2). In contrast, when we maintained BFN levels constant at 100 dB SPL, and varied playback attenuations between 10 and 30 dB, we found no effect on call frequencies ($P > 0.05$, df = 2, $\chi^2 = 5.19$, Kruskal–Wallis test; Fig. 3C₂).

The time courses for the call frequency rises were as fast as those for increases in amplitude. Call frequencies had already risen virtually by their maximum amount in the first call emitted after BFN onset (Fig. 3D). The frequency difference between the last call emitted before and the first call after BFN onset yielded the same significance level as when averaged over the last and first 10 calls ($P < 0.001$, df = 19, $\chi^2 = 156.9$, Kruskal–Wallis test with post hoc sign rank test; Fig. 3D). Similar to call amplitude, the frequency rise was not based on random fluctuations before or after BFN onset. Call frequencies were stable within the last 10 calls before and within the first 10 calls after BFN onset, showing no significant differences between each other [$P > 0.1$, df = 9, $\chi^2 = 3.66$ (before) 5.53 (after) Kruskal–Wallis test].

No Correlation Between Shifts in Call Amplitude and Frequency. Finally, we tested the relationship between the Lombard effect and the associated change in call frequency to see whether shifts in call amplitude and frequency varied with each other or whether they were modulated independently. We did not find any significant

Fig. 3. Changes in resting frequencies in response to BFN presentation. (A) Example of changes in vocal behavior in response to BFN presentation (condition 4). Spectrograms are plotted for a series of echolocation calls around BFN onset. The bat shows a shift in call frequency of 253 Hz in response to BFN presentation. (B) Shifts in call frequency in response to different BFN conditions (BFN amplitude: 100 dB SPL; playback attenuation: 30 dB) compared with no BFN stimulation; data averaged for all three bats. Medians: horizontal lines inside boxes; first and third quartile: upper and lower margins of boxes, respectively; 5% and 95% quantile: small horizontal bars above and below boxes, respectively. Dotted lines indicate the thresholds of the population effect size (±60 Hz). (C) Effects of different BFN intensities (C₁) and playback attenuations (C₂) on call frequency. Shifts in call frequency are shown in relation to shifts in response to BFN at 100 dB SPL in C₁, and relative to 30 dB playback attenuation in C₂. Data averaged for all three bats and all conditions. (C₁) Shift in call frequencies is slightly lower for weaker noise amplitudes (playback attenuation constant at 30 dB). (C₂) Stronger playback attenuation has no effect on shifts in call frequencies. (D) Changes of call frequency of the last 10 calls prior and the first 10 calls just after BFN presentation relative to the call frequency of the 10th call before noise onset. Data averaged for all three bats and noise conditions 2–6 (30 trials; medians with first and third quartile). First calls uttered after noise onset show a significant increase in call frequency (Kruskal–Wallis with post hoc signed-rank test).
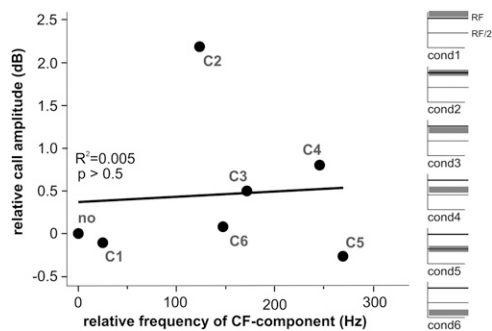
Fig. 4. Distribution of medians of call amplitude and relative frequency shifts of the CF component during the different BFN conditions and the control (no BFN; no). There is no significant correlation between call amplitude and the relative shift in call frequency in response to BFN (Pearson's correlation).

correlation between median call amplitude and the median relative frequency of the CF component for any of the BFN conditions tested (Pearson's correlation, $P > 0.5$; Fig. 4), indicating that amplitude and frequency shifts occurred independent of one another.

Discussion

We examined the Lombard effect and the associated change in call frequency in a highly vocal mammal—echolocating horseshoe bats—by analyzing how BFN that we presented at different frequency bands affected their echolocation behavior. We found that call amplitudes increased only when BFN was centered on the CF component of the bats' calls. This frequency range represents the bats' dominant call frequency and is quintessential for echolocation (24, 29–31). We found the strongest rises in call amplitudes for high BFN amplitudes. Such involuntary increases in call amplitude in response to increases in environmental noise have been observed in a large variety of animals (2–17), including humans (2). Most work on this Lombard effect used broadband stimuli to mask large portions of the animals' hearing range and therefore leaves open the question of whether specific frequency bands are important to elicit this effect. Only a few studies in birds (3, 7) and monkeys (17) found that masking noise yields the strongest Lombard effect when it covers the frequency range of the animals' calls. We show in horseshoe bats that call amplitudes exhibit robust shifts only when BFN masked the calls' dominant frequencies; even BFN extending up to 500 Hz below the dominant call frequencies did not have any significant effect.

In contrast to effects on call amplitudes, noise-dependent shifts in call frequency are not well-studied and relatively poorly understood (2–7, 9, 10, 12–16). Only two studies in birds reported a rise in call frequencies with increasing amplitudes of background broadband noise (8, 11). Similar to work on call amplitudes, it is unclear if such rises in call frequency occur uniformly across the hearing range of these birds. The data presented here, however, demonstrate that at least in echolocating horseshoe bats, the frequencies of echolocation pulses increased significantly for all but one frequency band masked by the BFN. Due to the population effect size, only one condition (condition 1, when BFN was presented just above the bats' RFs) did not show a significant effect of BFN on call frequencies, which was mainly due to large interindividual differences. This finding was notably different from the effect on call amplitudes, which was—also due to population effect size—limited to only one frequency band. As expected, the rise in call frequencies was stronger for larger BFN amplitudes.

In summary, our data demonstrate that the Lombard effect, i.e., a rise in call amplitude, and the associated rise in call frequency were affected by different frequency bands, an effect that has not,

to our knowledge, been reported previously. Whereas call amplitudes rose only when we masked the calls' dominant frequency components around RF with BFN, BFN affected call frequencies over most of the hearing range of horseshoe bats, which suggests different underlying neural mechanisms and circuits.

We also found that the rises in both amplitude and frequency already occurred in the first call emitted after BFN onset. This extremely fast latency for both parameters suggests that the underlying neuronal network can function without direct auditory feedback from the bat's own voice, which indicates a direct connection between the auditory and vocal-motor systems at the brainstem level. Earlier studies showed that auditory stimulation with clicks, pure tones, or noise evoked rapid reflex responses in the recurrent laryngeal nerve and the spinal ventral root with latencies between 6 and 25 ms (15, 32–36). Considering that call durations are typically between 40 and 50 ms, and calls are repeated every 50–150 ms, these studies suggest that auditory inputs originating from centers early within the ascending auditory pathway could directly affect laryngeal and expiratory muscles.

Though such direct feed forward loops at the level of motor neuron pools might be sufficient to explain the Lombard effect in most vertebrates, it appears to be somewhat more complex in horseshoe bats. The basis for the bats' remarkable echolocation ability is provided by specializations within their auditory system. A filter mechanism that is narrowly tuned to the echo's narrowband frequency component rejects background clutter while it helps to detect acoustic glints. This auditory filter is found in the cochlea, and results in an increased number of receptor cells in the cochlea as well as higher-order auditory neurons that are highly sensitive to the echo pure tone (27, 37). Because in the mammalian eye and within the visual central nervous structures the area of the optic fovea is overrepresented in a very similar manner, the auditory filter in horseshoe bats is similarly called an "auditory fovea" (29). During flight, the dominant CF components of the horseshoe bats' calls are shifted as a result of Doppler effects, and they compensate for these shifts by adjusting the frequency of the subsequent calls (24). This so-called Doppler-shift compensation (DSC) behavior ensures that the echoes remain within the bat's auditory fovea (27, 29). Returning echoes also vary in amplitude as a result of varying distances to objects. Bats compensate for these changes by adapting the amplitude of the successive calls to prevent echo intensities from exceeding an optimal level (38–40). Additionally, echolocating bats that use frequency-modulated (FM) sonar pulses for echolocation shift their call frequencies upon receiving ambiguous echoes (41). Therefore, feed-forward loops lacking any connections to the vocal pattern-generating system would not yield the Lombard effect with its associated changes in call frequencies that we observed in DSC and intensity-compensating bats. Instead, the bats would immediately compensate for any shifts in call frequency and amplitude. Consequently, the shifts in amplitude and frequency that we found have to be implemented within the neuronal network involved in vocal motor control to avoid such reflectory compensation. Several multimodal structures have been identified within the brainstem of bats and other mammals that may serve as candidates for audiovocal integration (42), such as the ventrolateral reticular formation (43, 44), the parabrachial region (45), the ventral nucleus of the lateral lemniscus (46, 47), the paralemniscal area (48, 49), and the external nucleus of the inferior colliculus (50). Further studies will tackle the questions of if and how these structures are involved in the neural adjustments governing the Lombard effect and the associated frequency changes.

In conclusion, we showed that auditory masking by BFN affected vocal behavior in echolocating horseshoe bats by shifting both call amplitude and frequency upward. Whereas call amplitudes rose only when we masked the dominant call frequency component around RF, call frequencies increased for most BFN conditions, which suggests that shifts in call amplitude and frequency are controlled independently. In addition, rises in amplitude as well as

in frequency were extremely fast and already occurred in the first call uttered after BFN onset, suggesting that the bats did not need auditory feedback of their own voice to control these changes. It appears, therefore, that the Lombard effect and the associated changes in call frequency involve (at least) two different neuronal mechanisms, which control call amplitude and frequency separately during audiovocal processing. Auditory neurons in horseshoe bats are exceptionally sharply tuned around RF (auditory fovea) (51, 52). Consequently, masking of echolocation calls is expected to be particularly strong when BFN maximally overlaps with RF (condition 2), which matches the effects we observed in call amplitudes. In contrast, BFN masking caused call frequencies to rise virtually uniformly across most of the bats' hearing range, with one notable exception. When we presented BFN above RF (condition 1), it appeared to have no significant effect when we averaged over all three bats. This result, however, may have been caused by large differences between the individual bats and therefore obscured the role of the auditory fovea for frequency changes associated with the Lombard effect. When we analyzed the effects of condition 1 on call frequencies in each of the bats separately (Fig. S1), we found that each of them responded differently: bat 1 decreased its call frequency, thus shifting it away from the interfering noise, bat 3 did not shift its frequency at all, and bat 2 increased its call frequency, thus actually shifting its call frequency closer toward BFN instead of avoiding the masking. These somewhat inconclusive results for BFN condition 1 may therefore suggest that the bats have several ways to regulate call frequencies when ambient noise falls into the auditory fovea.

Nevertheless, BFN presentation at different frequencies appeared to have a fairly uniform effect on call frequencies in all but one BFN condition, implying a rather general underlying neural mechanism. In contrast, the effects on call amplitude were somewhat less uniform, which may reflect a slightly larger diversity in the neural mechanisms used to overcome masking by environmental noise. Recently, different views have emerged on whether animals would use call amplitude or frequency to increase signal-to-noise ratios during masking by ambient noise (23, 53). In our study, the signal-to-noise ratio was significantly improved by an increase in call amplitude only when BFN masked the calls' dominant frequency. In contrast, shifts in call frequency did not increase the signal-to-noise ratio (condition 1 and 2), or increased it only slightly (condition 3–6). These results suggest that at least in horseshoe bats, shifts in call amplitude appear to improve signal transmission in noise during audiovocal communication more reliably than frequency shifts.

Our findings may aid in a better understanding of one of the most fundamental audiovocal phenomena, the Lombard effect, and thus reveal mechanisms of audiovocal integration and vocal motor control in a different light.

Materials and Methods

Animals. We used three greater horseshoe bats (*Rhinolophus ferrumequinum*), two males and one female, collected in the People's Republic of China, in this study. All animal experiments were carried out according to the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (54), and approved by the University of California, Los Angeles, Animal Research Committee.

Acoustic Stimuli. The bats called spontaneously and at RF. We acoustically stimulated the animals with playbacks of their own calls (echo mimics) that were not shifted in frequency (i.e., not eliciting any DSC) and, simultaneously, with continuous BFN. We generated BFN digitally using Tucker-Davis Technologies (TDT) system III hardware and OpenEX software. BFN was produced by digitally bandpass-filtering broadband noise to a bandwidth of 20 kHz with sharp flanks (bandwidth of BFN stimuli at −10 dB: <21 kHz). The 20-kHz BFN noise stimuli were centered around different frequencies as indicated in Fig. 1: condition 1, 10.5 kHz above the bat's RF; condition 2, at the bat's RF; condition 3, 10.5 kHz below the bat's RF; condition 4, 20 kHz below the bat's RF; condition 5, at the fundamental frequency of the bat's echolocation

pulses; and condition 6, at 20 kHz. These six conditions covered most of the bats' hearing range and thus allowed us to test the effects of noise on frequencies relevant for different behaviors, such as calling at rest (at RF, condition 2), listening to Doppler-shifted echoes (conditions 1 and 2), listening to echolocation calls emitted during DSC (conditions 3 and 4), listening to communication calls (condition 5), and listening to low-frequency ambient noise, such as noise caused by raindrops falling on vegetation (condition 6). As a control, we analyzed the bats' echolocation behavior without presentation of BFN (no BFN).

BFN stimuli had amplitudes of 80, 90, and 100 dB SPL. Pure tone and narrow-band noise stimuli (100–1,000 Hz) were not used because previous work (55) and our own pilot data showed that horseshoe bats immediately compensate to them. All BFN stimuli were presented under acoustic free-field conditions through an electrostatic speaker (ED1; TDT), which was placed ~20° laterally and 10 cm in front of the bat's left ear.

Echo playbacks (echo mimics) were generated as described previously (30, 31, 45). Briefly, echolocation pulses were captured by a calibrated 1/4-inch microphone (4939 with preamplifier 2633; Brüel and Kjær) placed 15 cm ahead of the bat's head. These calls were then played back with a 4-ms delay (produced electronically with TDT system III hardware and the OpenEX software) through an ultrasonic loudspeaker positioned right next to the electrostatic speaker presenting the BFN stimuli (Fig. 1A). This system allowed us to generate echo mimics by playing back calls at different attenuations. Playback attenuations used in this study were 10, 20, and 30 dB relative to the intensity of the echolocation pulses. Acoustic self-stimulation of the bat by its own calls has been shown to be approximately −26 dB below sound pressure levels measured in front of the pinna (56).

For acoustical stimulation, the bats were mildly restrained in a foam sandwich, but the animals' heads remained mobile. During call production, we continuously monitored the bats visually to ensure that the animal's head was directed straight ahead toward the microphone during vocal recordings. Generally, head movements were rare and occurred equally for all conditions tested. Therefore, potential effects of head movements were evenly distributed throughout all conditions, which may have resulted in blurring the entire data set. The call amplitude changes we observed represent a conservative estimate and may have been even more pronounced in animals with immobilized heads. In addition, whenever head movements occurred, we repositioned the head with a soft cotton swab back to its default position (Fig. 1B).

Echolocation pulses were recorded during different playback attenuations and/or different BFN amplitudes. Each of the different playback and noise conditions were presented pseudorandomly for 30 s at RF. Each condition was tested three times during a single experimental session (one session per day) for four different nonconsecutive days.

Analysis. All incoming signals were digitized with a CED Micro1401 mk II system (Cambridge Electronic Design) and recorded with Spike 2 software. Hereby, signals from the calibrated 1/4-inch microphone were digitized with 16-bit resolution and a sample rate of 200 kHz. Custom-made software (MATLAB; MathWorks) was used to measure call frequency and amplitude within the echolocation calls, and to detect call onsets and offsets. For call frequency measurements, we calculated a fast Fourier transformation (8,192 points; frequency resolution: 24.4 Hz) for every call and determined its peak frequency between each call onset and offset. Sound levels of the recorded playback noise were determined for all BFN conditions and subtracted from the call amplitude measurements taken for each echolocation call using logarithmic computation procedures (57). This method to determine call amplitude within ambient noise is well established and has been used in several previous studies on the Lombard effect (12, 58).

Statistics. Statistical analysis was performed with MATLAB (MathWorks Statistics Toolbox). We performed a one-way analysis of variance (Kruskal–Wallis test) with post hoc bootstrap analysis (10,000 repetitions) to test for significant differences in shifts in call frequency and amplitude within all BFN conditions. If the probability of error was less than 5%, we performed post hoc bootstrap analyses using Monte Carlo simulations to determine differences between each condition. We chose test statistics based on medians because they are more resilient to outliers and do not rely on normal distributions (call parameters were nonnormally distributed, Monte Carlo simulation). For post hoc bootstrap analysis, we calculated the median and median absolute deviation from a random sampling of 200 vocalizations (with replacement) in each condition. A set number of 200 vocalizations was used to remove the weighting of data toward a bat that vocalized more in a particular condition. This task was performed over 10,000 repetitions to produce a distribution of test statistics and followed by constructing 95% confidence intervals. We

determined that a distribution was significantly different if the 95% interval did not overlap with that of a different condition. To evaluate the relevance of different levels of statistical significance for changes in the bats' behavior, we calculated the population effect sizes (defined as 0.8 times of the SD to determine large effects). This parameter has been previously used to determine whether differences are present between two median values (59).

Pearson's correlation was used to examine the correlation between shifts in call frequency and amplitude. Correlations and differences in distributions were considered significant if the probability of error was less than 5%.

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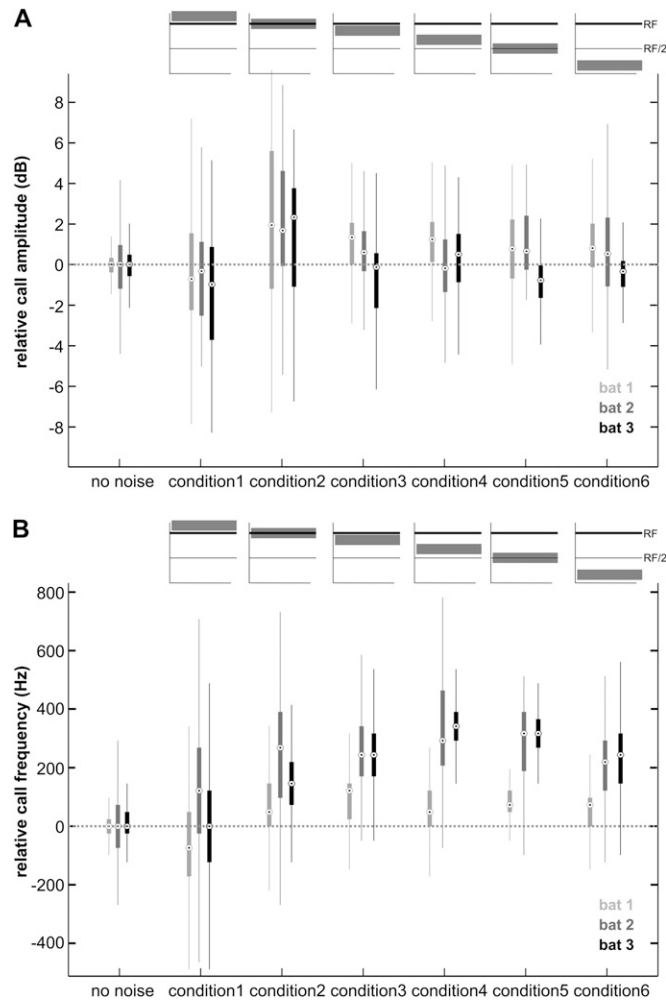# Supporting Information

Hage et al. 10.1073/pnas.1211533110

Fig. S1. Changes in call amplitude and frequency in response to BFN presentation compared with the control (no noise) for every individual bat. (*A*) Shifts in call amplitude were strongest for condition 2 in all three bats and were above the population effect size compared with the control [*P* < 0.001 for each bat, df = 6, χ^2 = 480.7 (bat 1), 187.1 (bat 2), 772.6 (bat 3), Kruskal–Wallis with post hoc bootstrap analysis for medians]. Thresholds of the population effect size were ±0.9 dB (bat 1), ±1.5 dB (bat 2), and ±1.2 dB (bat 3). (*B*) Shifts in call frequency for different BFN conditions compared with the control. BFN amplitude: 100 dB SPL; playback attenuation: 30 dB. Call frequency increases in all three bats for conditions 2–6 [*P* < 0.001 for each bat, df = 6, χ^2 = 814.3 (bat 1), 981.2 (bat 2), 3281.0 (bat 3), Kruskal–Wallis with post hoc bootstrap analysis for medians]. Interindividual differences occurred only during condition 1. Thresholds of the population effect size were ±33 Hz (bat 1), ±86 Hz (bat 2), and ±51 Hz (bat 3). Medians: black dots inside vertical bold bars; first and third quartile: upper and lower end of vertical bold bars, respectively; 5% and 95% quantile: end of horizontal lines above and below vertical bold bars, respectively.

Table S1. Shifts in call amplitude and frequency in response to different BFN conditions compared with control

BFN condition	Relative changes in call amplitude, dB			Relative changes in call frequency, Hz		
	Median	First quartile	Third quartile	Median	First quartile	Third quartile
No BFN (control)	0	−0.60	0.51	0	−48.8	48.8
BFN condition 1	−0.10	−2.39	1.87	24.4	−97.7	122.1
BFN condition 2	2.18	−0.93	4.07	122.1	48.8	219.7
BFN condition 3	0.50	−1.30	1.75	170.9	97.7	293.0
BFN condition 4	0.79	−0.75	2.07	244.1	97.7	366.2
BFN condition 5	-0.26	−1.30	0.85	268.6	97.7	341.8
BFN condition 6	0.08	−1.17	1.39	146.5	73.2	268.6

BFN amplitude: 100 dB SPL; playback attenuation: 30 dB; data averaged for all three bats (compare Figs. 2*A* and 3*B*).

Table S2. Effects of different BFN intensities and playback attenuations on call amplitude and frequency

	Relative changes in call amplitude, dB			Relative changes in call frequency, Hz		
	Median	First quartile	Third quartile	Median	First quartile	Third quartile
Constant playback attenuation						
80 dB	0.47	−1.21	2.11	−97.7	−170.9	−24.4
90 dB	0.88	−1.15	2.45	−48.8	−146.5	24.4
100 dB	2.18	−0.93	4.07	0	−73.3	146.5
Constant BFN amplitude						
10 dB	1.32	−0.20	2.51	0	−97.7	122.1
20 dB	0.66	−1.39	2.14	0	−99.5	146.5
30 dB	2.18	−0.93	4.07	0	−97.7	146.5

Call amplitude exclusively for condition 2; call frequency pooled for all BFN conditions; data averaged for all three bats (compare Figs. 2*B* and 3*C*).