Article

Doppler detection triggers instantaneous escape behavior in scanning bats

Doppler shifts were enough to induce escape behavior

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Highlights

We demonstrated how echolocating horseshoe bats "see" approaching objects

Only the Doppler shifts, not the temporal changes in echo delay, acted as a cue

Velocity is directly perceived, not temporal changes in position as in vision

Taking advantage of acoustic sensing, the bats enabled instantaneous threat avoidance
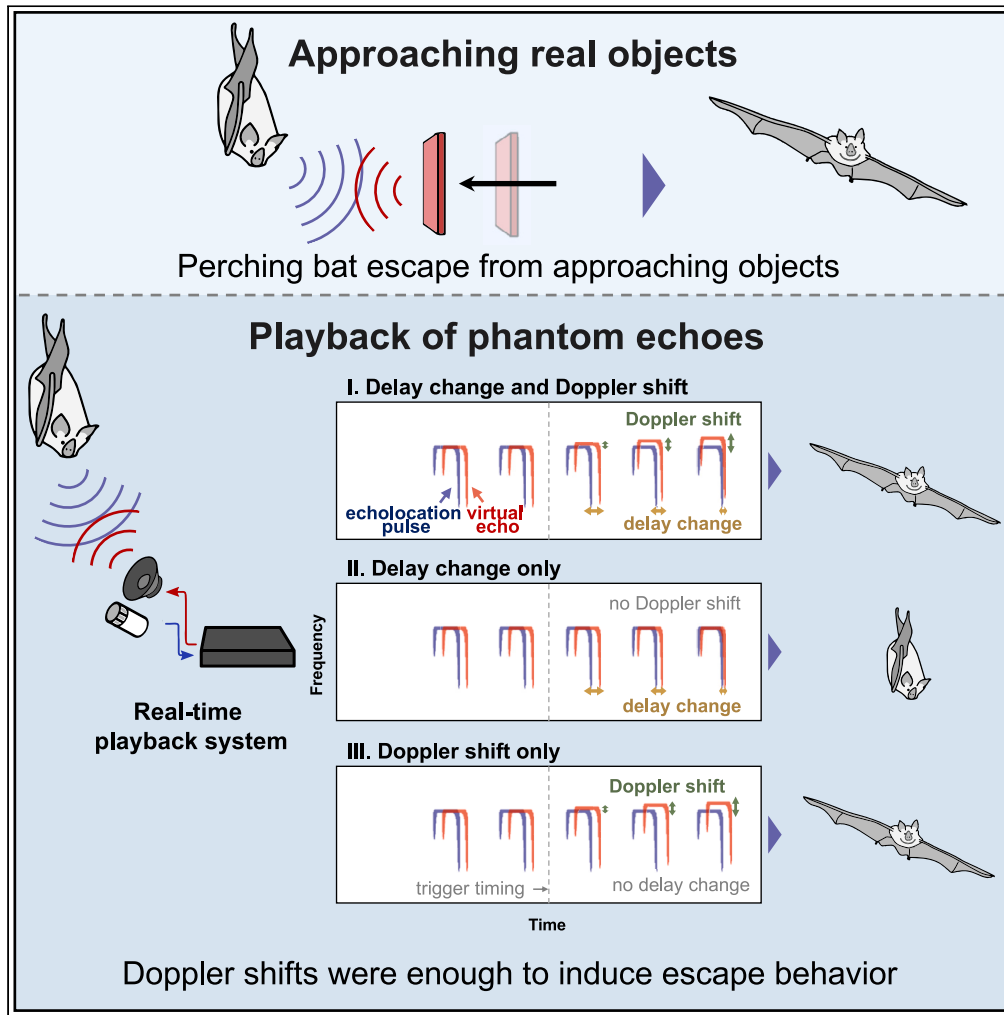
Article

Doppler detection triggers instantaneous escape behavior in scanning bats

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SUMMARY

Animals must instantaneously escape from predators for survival, which requires quick detection of approaching threats. Although the neural mechanisms underlying the perception of looming objects have been extensively studied in the visual system, little is known about their auditory counterparts. Echolocating bats use their auditory senses to perceive not only the soundscape, but also the physical environment through active sensing. Although object movement induces both echo delay changes and Doppler shifts, the actual information required to perceive movement has been unclear. Herein, we addressed this question by playing back phantom echoes mimicking an approaching target to horseshoe bats and found that they relied only on Doppler shifts. This suggests that the bats do not perceive object motion in the spatio-temporal dimension (i.e., positional variation), as in vision, but rather take advantage of acoustic sensing by directly detecting velocity, thereby enabling them to respond instantaneously to approaching threats.

INTRODUCTION

To survive, animals must take appropriate action after detecting prey or predators in a constantly changing environment. Abrupt changes in the sensory input can indicate the presence of prey or predators.[1] Distal senses such as vision and audition are particularly important for this purpose because these sensory modalities provide an animal with the presence and/or movement of an object located far away in real time. The neural mechanisms underlying the perception of approaching threats have been extensively studied in the visual system,[2,3] but little is known about those in the auditory system. Previous studies have revealed that acoustic looming stimuli mimicking approaching sources are perceived as salient in primates[4,5] and elicit defensive behaviors in rodents.[6,7] However, there is limited knowledge regarding the extent to which natural sounds trigger escape behavior in vertebrates.

Echolocating bats provide useful models for studying how auditory sensory inputs modulate their behavior because the spectral and temporal patterns of their vocalizations reflect changes in their behavioral state.[8] They can compare self-generated signals and/or reflections from static objects with abrupt changes in reflections from a moving object.[9] Therefore, they can detect approaching objects by using more cues than passively listening animals, such as changes in the frequency and delay of echoes, to respond rapidly to approaching threats. However, despite their importance, it remains unknown which acoustic characteristics of returning echoes play a critical role in detecting moving objects for rapid escape behavior.

Japanese horseshoe bats (*Rhinolophus ferrumequinum nippon*) emit ultrasound pulses characterized by a long constant-frequency (CF) component followed by a brief downward frequency-modulated (FM) component, which is sometimes preceded by an upward FM component.[10] In CF-FM echolocating bats, the target range, the key perceptual factor obtained through echolocation, is measured from the time interval between the terminal FM components of an emitted pulse and the returning echo, or echo delay.[11–13] In addition to echo delays, Doppler shifts in the echo CF frequency are crucial in CF-FM bats. These bats cancel out flight-induced Doppler shifts by modifying the emitted pulse CF frequency to maintain the echo CF frequency within a narrow frequency bandwidth, to which their auditory system shows extreme sensitivity.[10,14,15] This behavior, termed Doppler shift compensation, is believed to play a critical role in detecting insect fluttering in the presence of vegetation.[1,9]

Understanding how bats perceive motion provides comparative insights into how different sensory modalities respond to the same physical phenomenon, which is essential for a comprehensive understanding of how animals perceive the world. However, the information required to perceive object movement in bats remains unclear. Since echoes coming back from an approaching object are theoretically characterized by both changes in echo delay and the presence of Doppler shift, and CF-FM bats are sensitive enough to both temporal and frequency information, we hypothesize that they use both or either the "delay change" and "Doppler shift" information. If this is the case, acoustic

manipulation of both "delay change" and "Doppler shift" or either of them should be able to induce an equivalent response in the bats to the real approaching object. To test this hypothesis, we conducted phantom echo playback experiments to simulate a moving object by manipulating the two acoustic cues.

RESULTS

Preliminary experiment: Confirmation of response to approaching objects

First, we conducted an exploratory experiment in which we presented a real moving object to bats hanging on a perch to examine how horseshoe bats react when they detect an approaching object. A spring-loaded plastic panel (0.45 × 0.45 m) was moved toward the perching bats from 1.5 to 1.0 m over 200 ms in an experimental flight room (Figure 1B; Video S1). It was observed that the bats changed the properties of their echolocation calls and then displayed takeoff behavior immediately after the panel started to approach them. Specifically, they decreased the duration of CF components and increased the FM bandwidth (Figure 2A). Since horseshoe bats showed a rapid takeoff from a perch and changed the properties of their echolocation calls in response to the approaching object, we could evaluate the bats' reactions to moving objects by utilizing this response without any training.

Phantom echoes induced the same response as real approaching objects

For the next step, a real-time playback system capable of signal processing with a delay of approximately 1 ms was used to simulate a virtual approaching object, and the movement of the real object we had presented to the bats was acoustically mimicked. In the phantom echo experiments, nine *R. f. nippon* bats that demonstrated takeoff behavior in response to a real approaching object were used, so we could assume that the phantom echo was recognized as an approaching object if the bats showed the same takeoff response. A microphone placed 1.0 m away from the bat was used to pick up the emitted echolocation sounds from a perching bat. After real-time signal processing for approximately 1 ms, the sounds were delivered back to the bat as phantom echoes from the simulated object using a loudspeaker beside the microphone (Figure 1A). After a few seconds of presenting the virtual static object with a 4 ms delay, the echo delay was gradually changed from 4 to 1 ms over 200 ms, corresponding to an approach from 1.7 to 1.2 m. Simultaneously, the frequency shifts in the phantom echoes were changed by heterodyning from 0 (i.e., no Doppler shifts) to 1.6 kHz, which were approximately equivalent to the changes of the position and velocity in the real-approaching-object experiment (I in Figure 1B). Phantom echo amplitudes were adjusted to reproduce approximate echo amplitudes from the plastic panel used in the previous real-approaching-object experiment.

As a result, the tested bats responded to the virtual moving object in a manner similar to their responses to the real moving object. The bats decreased the pulse duration and increased the bandwidth of the FM component when the virtual object approached, and seven of the nine tested bats showed takeoff behavior (I in Figure 2 and Table 1; Videos S2 and S3). Overall, by exposing the bats to virtual objects that mimicked a real target's approach by manipulating both the Doppler shifts and echo delays, we successfully observed takeoff behavior and changes in the acoustic characteristics of the emitted pulses, as observed in the real target's approach.

Not changes in echo delay but only Doppler shifts were sufficient for the response

Next, we examined which acoustic manipulations in the phantom echoes induced takeoff behavior. The bats were tested under two additional conditions, wherein only the echo delays or Doppler shifts in echo CF (II and III in Figure 1B and Table 1) were manipulated. Surprisingly, none of the nine tested bats showed clear changes in the emitted sounds or escape behavior in response to the phantom echoes with echo delay decreasing (II in Figure 1 and Table 1; Video S4). However, positive Doppler shifts in echo CF alone evoked changes in the duration and the FM bandwidth of the emitted echolocation pulses, and seven of the nine tested bats showed takeoff behavior (III in Figure 1 and Table 1; Video S5). These results indicate that only the Doppler shifts are necessary and sufficient for horseshoe bats to elicit takeoff behavior while scanning their surroundings during perching.

Fast responses with higher intensity phantom echoes

We then focused on the latency of the reactions being so short that they had already started to react within 200 ms of the presentation of the virtual object. These fast reactions are crucial in the context of escape behavior and are perhaps a benefit of focusing on the Doppler shift. Hence, more intense stimuli were used to further investigate the maximum response speed of bats. The phantom echoes were presented at a 20 dB higher amplitude than in the aforementioned experiments. In high-intensity playback experiments, six tested bats took off from the perch in response to Doppler shifts; however, only one of seven bats took off in response to the phantom echoes with echo delay decreasing (Table S1A), which confirmed that the Doppler shifts were necessary and sufficient for the detection of approaching objects. Regarding responses to echolocation calls, they consistently showed reactions in response to a Doppler shift alone more rapidly than in response to phantom echoes with a lower amplitude. In detail, the pulse duration was already decreased in the second pulse after the trigger signal, and surprisingly, the FM bandwidth was increased at the very first pulse after the trigger input (Figure 3). This suggests that these bats can adjust the characteristics of the FM component of a pulse while listening to the shifts in the CF of the echo of the same pulse. Considering that the mean duration of the pulse was approximately 40 ms and the echo delay at the bats' position was 10 ms, their reaction time to approaching objects could be less than 30 ms based on the increased bandwidth of the FM component.
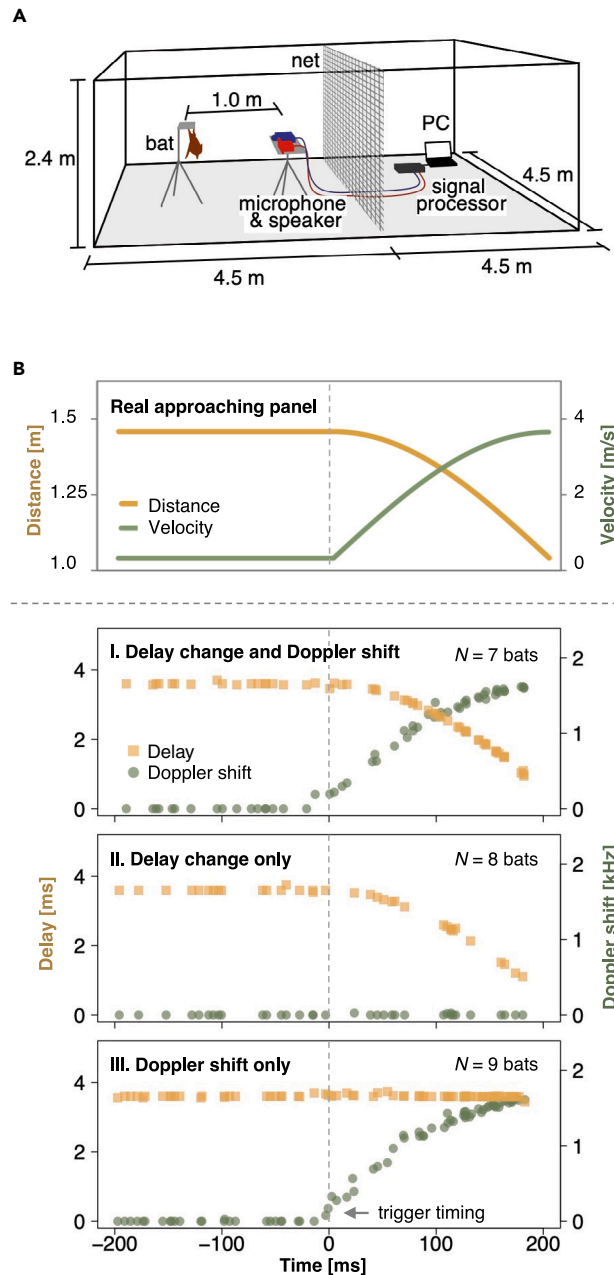
Figure 1. Experimental setup and playback stimuli

(A) In an experimental flight room, bats were placed on a perch 1.0 m away from a microphone and a playback speaker. Using this real-time playback system, we manipulated the echo delays and/or the Doppler shifts of the emitted echolocation pulses and played them back as phantom echoes.

(B) Actual values of the echo delays and the Doppler shifts of three types of phantom echoes. The echo delays and Doppler shifts were programmed to reproduce the motion of the approaching panel in the preliminary experiment, based on its distance and velocity changes. Both were successfully manipulated to reproduce an approaching virtual object. Due to some technical errors that occurred in the experiments, three of the 27 trials were excluded from the figures and following data (see also Figure S2). The success rate of the acoustic feature control was 89% (24/27).

DISCUSSION

In terms of echolocation, object motion is reflected in both the Doppler shifts of the echoes and changes in the echo delay, making it theoretically possible for bats to use both of these types of information in the perception of approaching objects. For visual motion perception, a model for motion detection based on the spatial-temporal tilt of the image produced by a moving object has been proposed and confirmed it at the neuronal level.[16] If this model were to be applied to the auditory system, it would be reasonable to predict a model that detects motion
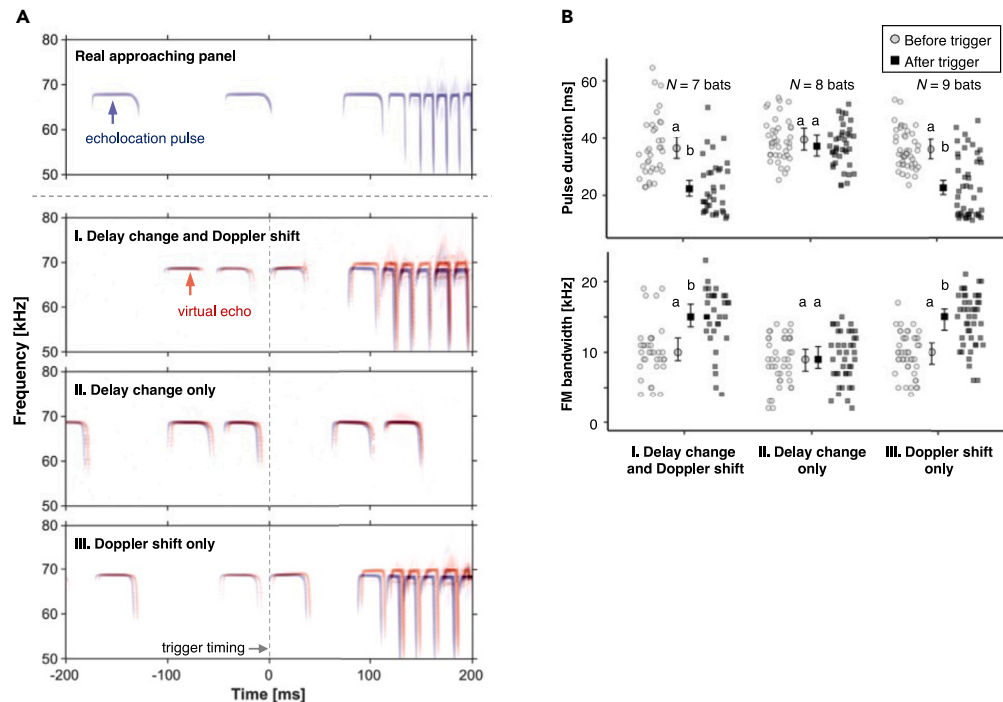
Figure 2. Responses to the phantom echoes

(A) Spectrograms of representative echolocation pulses (blue) and phantom echoes (red) (Bat ID: 2695).

(B) Changes in pulse duration and frequency modulation (FM) bandwidth in response to a virtual approaching object. In response to stimuli I and III, pulse durations decreased and FM bandwidth increased ($p < 0.0001$, after p value adjustment using the Bonferroni method), whereas there was no significant response to stimulus II. Significant differences between the values are indicated by different letters. The individual means of each acoustic feature calculated for each individual from three pulses before the trigger input are indicated by gray circles, and the means calculated from three pulses after the trigger are indicated by black squares. Mean values and 95% confidence intervals are represented by plots and bars, respectively. See also STAR methods and Tables S2 and S3.

based on temporal changes in the echo delay, an acoustic feature used for object ranging.[11] However, surprisingly, the tested bats did not show any motor or vocal responses to the phantom echoes with only echo delay changing. Instead, the bats showed rapid responses, such as takeoff behavior and changes in the acoustic characteristics of emitted pulses, immediately after the virtual objects containing the Doppler shift information started to approach them. This indicates that CF-FM horseshoe bats do not use the echo delay but only the Doppler shift for motion detection, at least during perch sitting.

Horseshoe bats prey on flying insects from near or within vegetation in flight while hanging on a perch and searching for prey.[9,17] In this situation, the distance between the prey insect and the background vegetation is so small that the individual echoes from each leaf result in a complex acoustic scenario called clutter, wherein the target echoes can be masked by irrelevant echoes.[18] Unlike with human vision, which acquires two-dimensional information, echolocation relies on hearing and acquires one-dimensional information. This results in overlapping echoes from both a target and irrelevant objects, which may make it difficult to distinguish target echoes from the overlapping background echoes. In these complex clutter situations, it would be difficult to follow the movement of objects using relevant echo delays. Furthermore, echolocation, through which the surrounding environment is understood using intermittently emitted pulses, gives bats a discontinuous stroboscopic perception of the world and is not suited to the perception of continuous flow as is vision. Under such limitations faced by CF-FM bats in cluttered environments, a model that detects motion based on the time variation of an echo delay is unlikely to be robust.

The auditory fovea is thought to be an adaptation for detecting amplitude and frequency modulations in returning echoes caused by fluttering insects' periodic wing movements in clutter.[9,19] In other words, high duty cycle (HDC) echolocating bats already have a mechanism that can detect slight Doppler shifts with high precision to detect minute movements of the wings of flying insects that are practically impossible to detect owing to temporal changes in echo delay. Our present observations indicate that Doppler shift is consistently used even in situations where echo delay is available, such as detecting the dynamics of natural enemies, which supports previous findings that adaptation of the auditory system and behavior allows horseshoe bat specialists to detect Doppler shifts.[9,20] The auditory system of HDC bats, which has been refined to use Doppler shift for robust prey detection, helps them detect approaching threats. Furthermore, this suggests that Doppler shifts are more robust information for recognizing moving objects than changes in echo delay in clutter and that the auditory fovea detects abrupt movements, such as insect fluttering, and more global movements of objects to successfully capture insect prey or escape from predators in the presence of clutter. What have been discussed regarding the presence of auditory fovea for glints detection and the usefulness of

Table 1. Proportion of the bats showing the escape response

Bat ID	I. Delay & Doppler	II. Delay only	III. Doppler only
2670	fly	stay	fly
2680	stay	stay	stay
2681	fly[a]	stay	fly
2682	fly	stay	fly
2687	fly	stay	fly
2689	fly[a]	stay[a]	fly
2691	fly	stay	fly
2695	fly	stay	fly
2893	stay	stay	stay

Seven of the nine bats displayed takeoff behavior responding to stimuli I and III, and none responded to stimulus II.
[a]Manipulation of the Doppler shifts or delay did not work well (See also Figure S2).

Doppler shift in clutter environments is generally true not only for the horseshoe bats, but also for other HDC bats that emit echolocation calls with a CF component, such as hipposiderids.[9,21] Therefore, we hypothesize that the tactic of utilizing Doppler instead of changes in echo delay is similarly employed in the perception of approaching objects in other HDC bats. However, it is known that the accuracy of Doppler shift compensation behavior or the sharpness of the auditory fovea differs in HDC bats,[9,22] making it likely that the response and sensitivity to approaching objects differ. Therefore, further studies comparing and verifying this finding with other bat species are warranted.

Note that we confirmed that the bats flew away from the stimulus rather than approach it (Figure S1). Consequently, it is reasonable to posit that the response to the phantom echoes was not amount to an interested behavior triggered by the detection of fluttering prey but an escape behavior elicited by the recognition of an approaching threat.

The Doppler shifts triggered an instantaneous response that increased the bandwidth of the FM of the ongoing pulse with a reaction time of less than 30 ms. Fast escape responses have been studied in other animal models. Squid can respond to light stimuli in 50–75 ms[23]; zebrafish can respond to vibration stimuli in 3–5 ms[24]; and mice can initiate escape responses 250 ms[25] after visual enemy stimulus. Comparatively fast responses have also been reported in bat echolocation research. For example, the acoustic and behavioral reaction times of FM bats to various situational changes perceived by echolocation are 50–90 ms.[26–28] The responses to passive stimuli that do not depend on echolocation are even faster, with a response latency of only 30 ms for the Lombard effect,[29] and 20 ms has been reported for simple startle responses (startle response to a loud clicking sound).[26] The present results show acoustic responses to auditory input in the context of echolocation with a latency of less than 30 ms, indicating that CF-FM bats using different strategies for echolocation with a HDC and longer signal from FM bat species can also achieve comparable or even faster response times. This may be due to the ability of CF-FM bats to detect Doppler shifts, which allows them to perceive approaching objects in a single sensing cycle without the cost of processing multiple FM echoes of successive pulses. This must be advantageous for fast escape behavior, especially in CF-FM bats that spend most of their time perching on branches using a fly-catcher foraging style[30] since this strategy makes it easy for predators to find. Thus, they require swift threat detection. In addition to the response speed, it is worth mentioning that the bats increased the bandwidth of the FM component in the very first pulse after Doppler presentation. This demonstrates that the parameters of the FM component are not yet fixed at the time of vocalization of the CF component, or are at least adjustable in real time, and the feedback from the CF echoes can be reflected in the detailed parameters of the FM component even after the bat has started to emit the CF component. Such behavioral responses have not been previously known and support the hypothesis that the CF and FM components are independently controlled, as suggested by several studies.[31,32]

When a Doppler shift is detected, the bandwidth of the FM is increased, instead of an increase in the duration and intensity of the CF component to measure the frequency shift more accurately. This transition from the scanning phase to the ranging phase wherein the pulse duration decreases and the FM bandwidth increases further supports the importance of FM components for target ranging in horseshoe bats. In horseshoe bats, temporal overlaps between pulses and echoes are necessary for Doppler shift compensation.[33] Pulse duration may reflect how far the bats want to "see" through echolocation because the possible maximum temporal overlap between pulses and echoes is theoretically constrained by pulse duration. Additionally, the broader the bandwidth of pulses a bat emits, the higher the resolution of the target ranging could be.[11,34] Consequently, the rapid decrease in pulse duration and the increase in bandwidth in response to virtual moving objects are both possibly linked to their emergent necessity in accurately scanning surroundings, especially for nearby objects. This target-ranging behavior may be required to accurately determine the location of an approaching object and to obtain the information necessary to determine whether and in which direction to flee.

Therefore, to examine whether the escaping behavior changed depending on the delay, as an additional experiment, the delay was changed from 0 to 18 ms, which corresponds to 1.0–4.0 m, and the results showed that the response decreased as the echo delay increased (Table S1B). This indicates that for bats, the approaching object at medium distances (1.2–1.7 m) is recognized from the Doppler shift rather than the change in echo delay, but they tend not to run away at distances greater than approximately 3 m. Taken together, these results suggest that, although the Doppler shift in the CF component plays a crucial role in the initial detection of an approaching object, the echo delay
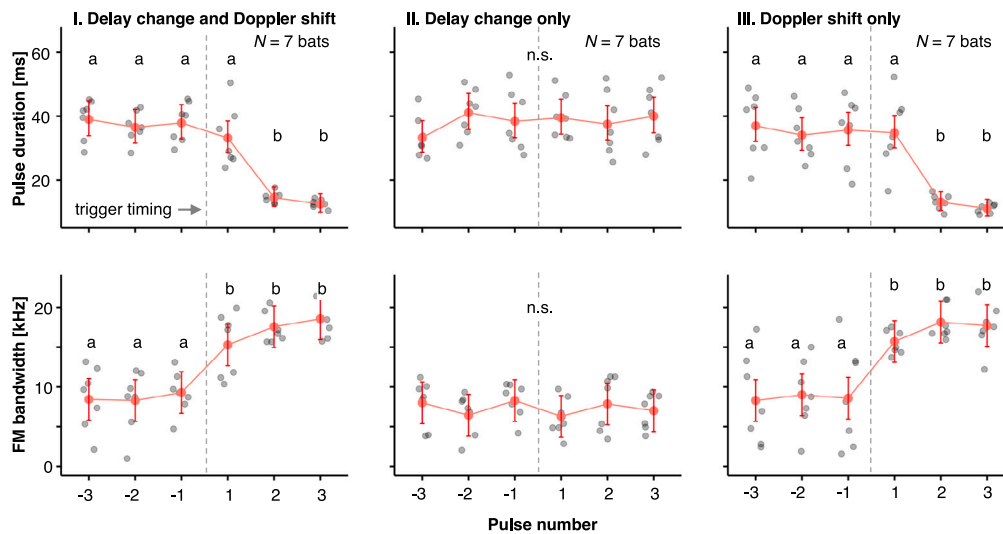
Figure 3. Rapid changes in pulse duration and FM bandwidth in response to the high-intensity playback experiment

The pulse duration started to decrease at the second pulse after the trigger input, and the FM bandwidth started to increase at the very first pulse after the trigger in response to high-intensity stimuli I and III ($p < 0.0001$, $p < 0.01$ each, after p value adjustment using the Bonferroni method), whereas there was no significant change in response to stimulus II. All individuals' data are indicated by gray circles. Mean values and 95% confidence intervals are represented by red plots and bars, respectively. See also STAR methods and Tables S2 and S3.

in the FM component plays an important role in the subsequent phases. Timing cues may be more important for bats when objects are closer together, such as within 1 m.

In summary, herein, we have demonstrated how object motion is perceived by bats, which are specialists in the auditory sense and actively perceive their surroundings through the ultrasonic pulses they emit and through the resulting echoes. The results indicated that horseshoe bats sitting on a perch relied on Doppler shifts in the echo frequency of the CF component, rather than on the temporal information in the FM component, for the initial detection of a moving object. The results further indicate that the bats use such changes in Doppler shift to respond rapidly. Although it was surprising that temporal changes in echo delay were not used, given the common understanding of the visual system, these findings demonstrate that different sensory modalities have completely different sensing mechanisms even for the same physical phenomenon, each taking advantage of the strengths of each sense.

Limitations of the study

While this study was on horseshoe bats, it would be interesting to conduct the same experiments with other CF-FM bats such as roundleaf bats to see if they use the frequency information in the same way. Furthermore, identifying the mechanism for the perception of approaching objects in FM bats, which are specialized in detecting the temporal information of echo delays, and comparing them between auditory specialists gives us comprehensive understanding of how echolocating bats perceive the approaching objects. However, we did not have other CF-FM bats that we could experiment with, and we have attempted the same experiments with one of the FM bats (*Miniopterus fuliginosus*) that we could experiment with, but they discontinued emitting echolocation calls once they perched on a perch, thus we could not test them in the same experimental paradigm of phantom echo presentation.

STAR★METHODS

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● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109222.

AUTHOR CONTRIBUTIONS

S.Y. and K.H.: conceptualization, methodology, software, and investigation. S.Y. and O.H.: formal analysis. S.Y.: data curation and visualization. K.H.: writing – original draft and review and editing. S.Y., O.H., K.I.K., and S.H.: writing – review and editing. S.Y., K.H., and S.H.: funding acquisition. K.I.K. and S.H.: supervision. S.H.: resources and project administration.

All authors gave their final approval of the manuscript for publication and agreed to be held accountable for the work reported therein.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Data and Code for Analyses	Mendeley Data	https://doi.org/10.17632/m6gtkn6hgj.1
Experimental models: Organisms/strains		
Japanese Horseshoe Bats (*Rhinolophus ferrumequinum nippon*)	Wild	N/A
Software and algorithms		
MATLAB R2021a	MathWorks	https://jp.mathworks.com/downloads/web_downloads
R 4.2.2	The R Foundation for Statistical Computing	https://cran.ism.ac.jp/
RPvdsEx	Tucker-Davis Technologies	https://www.tdt.com/support/downloads
Motive Tracker 3.0.1	OptiTrack	https://www.optitrack.jp/support/support01/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Kazuma Hase (kazuma.hase01@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The datasets generated during this study are available at Mendeley data (https://doi.org/10.17632/m6gtkn6hgj.1) and is publicly available.
- The original code is available at Mendeley data (https://doi.org/10.17632/m6gtkn6hgj.1) and is publicly available.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In this study, 15 adult Japanese horseshoe bats (*Rhinolophus ferrumequinum nippon*; seven males and eight females) were used. The exact ages of the bats were unknown because they were all wild caught. The bats were captured from a cave located in Fukui Prefecture, Japan. We were licensed to collect bats, and we complied with all Japanese laws (permits from Fukui Prefecture in 2019 and 2020). The bats were housed in a room (3 × 2 × 2 m) at Doshisha University in Kyoto, Japan. To mimic the cave environment, the temperature and humidity of the room were monitored and controlled. Every 2–3 days, the room was cleaned, and mealworms and water were placed to allow the bats to eat and drink *ad libitum*. The experiments were conducted under the permission of the Animal Experimentation Committee of Doshisha University (Permission No.: A20026).

METHOD DETAILS

Experimental system

An experimental chamber (9 × 4.5 × 2.4 m) was used to conduct experiments. A net was hung to separate the chamber into two compartments: one was the flight space for the bats (4.5 × 4.5 × 2.4 m), and the other was for the experimental equipment and researchers (4.5 × 4.5 × 2.4 m). There was acoustic foam on the walls to minimize echoes from the walls. During the experiments, long-wavelength lighting with red filters (filtering out wavelengths below 650 nm) was used to decrease the possibility of bats using visual information.[35] A perch made of wire mesh or net was installed 1.2 m from the floor using a tripod. When a bat flew away from the perch, we waited until it landed somewhere in the chamber, and then we gently caught it by hand or a butterfly net and placed it back onto the perch. We tested each individual once a day for each condition to investigate their naive responses to approaching threats.

Virtual object experiment

The phantom echoes from virtual approaching objects were artificially generated by convolving the echo delay and Doppler shifts into the echolocation pulses picked up by a microphone in real time using a digital signal processor (DSP, Tucker-Davis Technologies, RX6). To reproduce the movement of the object in real-object experiments, virtual objects were used. Because the real object was moved by the force of an extension spring, if the friction force is ignored for simplicity, it can be assumed that the movement is a quarter cycle of simple harmonic motion, which means that a trigonometric function of time can describe the position and velocity of the object. Considering that the real object moved 0.5 m in 0.2 s, the calculation showed that the distance changed from 0.5 to 0 m and that the velocity changed from 0 to 4 m/s. This corresponds to a change in the echo delay from 3 to 0 ms and a change in the Doppler shift from 0 to 1.6 kHz in 0.2 s, assuming that the speed of sound is 340 m/s and that the frequency of the echolocation pulse is 68 kHz. Based on this calculation, the DSP was programmed to change the playback timing and/or the frequency of the artificial echo from 3 to 0 ms and 0 to 1.6 kHz in 0.2 s, respectively, when the trigger signal is input. A double heterodyne method was used to achieve frequency modulation.[15] Briefly, to increase the playback frequency by x kHz, the echolocation calls were first multiplied by a 20 kHz pure tone signal, passed through a 70–90 kHz bandpass filter, and then multiplied again by a $(20 - x)$ kHz signal and passed through a 73–53 kHz bandpass filter. The frequencies of the multiplying signal and filter were determined based on the CF component frequency (68 kHz) and the FM component bandwidth (20 kHz at maximum). In the present study, we extracted only the second harmonic of echolocation calls to generate phantom echoes. This was because the double heterodyne method could not be performed with the full bandwidth of echolocation calls with the sampling rate of the real-time playback system. It is widely known from past behavioral and neurobehavioral studies on DSC behavior that HDC bats mainly use the CF component of the second harmonic for Doppler shift compensation,[9,21] for example, by manipulating the frequency of the CF2 component of the playback echo to induce DCS behavior.[36]

Next, we examined whether responses to virtual moving objects can be considered escape behaviors or not. To verify this, we placed speakers on either side of the perch and playbacked them randomly while measuring the flight path of bats and checking whether the bats flew away in the direction of escape from the speaker. Four chains were hung in front of the bats so that they flew only to the left or right. Stimuli were presented eight times to each of the two individuals, and a motion-capture system (Naturalpoint, Inc. DBA OptTrack, Motive Tracker) was used to measure flight paths. Consequently, although the bats flew to the front, they tended to move away from the speaker (Figure S1). This suggests that the response to virtual moving objects is not simply a reflexive response but an escape behavior from approaching objects.

Although most bats displayed takeoff behavior in response to the stimuli, "I Delay change and Doppler shift" and "III Doppler shift only," they did not take off in four trials. In these cases, the stimuli were often presented at a time when bats did not emit many echolocation calls (the top plot in Figure S2). In other words, the phantom echoes that bats could receive were few, suggesting that they were less likely to detect the virtual objects adequately, and this is likely the reason why the bats did not take off. We also observed that the bats did not take off but showed a startled response to the presented stimulus (Video S3), which was not seen in response to the "II Delay change only" stimulus (Video S2).

The playback system did not work well in 3 of 28 real-volume experiments (marked with asterisks in Figure 3C). Specifically, the Doppler amount or echo delay did not change as expected, mainly because of the low sound pressure of the bat's emitted pulses. Because changing the echo delay during the playback of the echoes would cause artifacts, it is necessary to change the echo delay between pulses and instantaneously stop the echo delay change when the bats are emitting pulses. For this reason, the playback system needs to detect the pulse timing from the amplitude, but in these trials, the bats' vocalizations were too low, and the system could not detect the pulses properly, resulting in a Doppler shift larger than the theoretical value shown in the bottom plot of Figure S2. Therefore, these data were excluded from other graphical and statistical data.

Sound and video recordings

Echolocation calls (input for the DSP), artificial echoes (output from the DSP), and the trigger signals were recorded with a PC in one file through a data acquisition system (National Instruments, USB-6356) at a sampling rate of 500 kHz. A high-speed camera (IDT Japan, Inc., MotionXtra NX8-S1) set on a camera tripod located 0.7 m away from the perch was used to monitor the physical reactions of the bats. Videos were recorded with 100 frames per second 0.5 s before and after the trigger. Because sounds and a trigger were recorded in the same file, acoustic data and video could be analyzed at the same time.

Acoustic and behavioral analysis

The timing and duration of the emitted pulse and the delay of phantom echoes were measured with an accuracy of 0.1 ms, and oscillograms of the recordings were created using acoustic software. Next, a custom-written MATLAB program was used to analyze the FM bandwidth and the Doppler shifts of each pulse and echo. The FM bandwidth was defined as the frequency band that is −20 dB relative to the peak intensity of the terminal FM component, and it was calculated using fast Fourier transform (FFT) with 1024-point windows, providing a resolution of 488 Hz. Doppler shifts were defined as the difference in peak frequency between the pulse and the echo and were calculated using FFT with the whole pulse as windows.

Behavioral responses were categorized into takeoff and stay-still behaviors based on video recordings. Because the videos were recorded until 0.5 s after the trigger input, takeoff was considered when bats reacted within 0.5 s after stimulation.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical software environment R was used to conduct all of the following analyses.[37] As outlined in the main text, this study aimed to find whether echolocation call characteristics differed between pulses emitted before and after the trigger. Additionally, three acoustic cues in the broadcast echoes were used to investigate which are most important for evoking a response in perching bats. In this study, we focused on pulse characteristics, such as the pulse duration and bandwidth of the frequency-modulated terminal part of the pulse. For this analysis, five pulses before and after the trigger were considered.

The duration of these pulses was modeled as a function of the broadcast status (before and after) in interaction with the acoustic cues used in the broadcast echoes (echo delay only, echo delay and Doppler shifts, and Doppler shifts only), including a random factor for the bat ID due to repeated testing. Because the pulse duration cannot be zero, generalized linear mixed models were applied using the Template Model Builder package in R,[38] and models were prepared using four zero-truncated families (nbinom1, nbinom2, genpois, and poisson) to find the best fit. Because of within-group heteroscedasticity, a term was added to the four models to estimate the dispersion of the interactive effect. The bandwidth data of the FM terminal part of the pulse were normally distributed, allowing for modeling using linear mixed models.[39] The bandwidth was modeled as a function of the same interactive effect as above while also including the bat ID as a random factor.

In the second step, we were interested in how quickly the bats could adapt their pulse characteristics after the trigger. For this, the pulse duration and bandwidth of the FM pulse part between each of the three pulses before and after the trigger were compared. Furthermore, we checked if changes among pulses would differ depending on the type of acoustic cue (echo delay only, echo delay and Doppler shifts, and Doppler shifts only). Thus, we modeled the pulse duration similarly as above as a function of the pulse number (six levels) in interaction with the acoustic cue type (three levels) using glmmTMB and the above-mentioned families as well as the bat ID as a random factor. Converging and well-fitting models were then compared based on their Akaike information criterion (AIC) values. The bandwidth of the FM pulse part was modeled in the same way as above, except that the data were modeled as a function of the pulse number in interaction with the acoustic cue type.

Generally, the quality of the model fit was checked based on residuals using various functions.[40] All of the final models were checked for their overall significance compared with their null models using a χ^2-test[37] for glmmTMB models and a parametric bootstrap method[41] for lmer models. A type II Wald χ^2-test was used to test the significance of single factors within each model.[42] Estimated marginal means were calculated for each model, and comparisons among factor levels were conducted within each group of the acoustic cue type while applying a Bonferroni correction for multiple comparisons.[43]

The residuals of all final models did not show any signs of model assumption violations. Regarding the model for call duration in the first experiment, only one (family: truncated_genpois) of the four models converged and fit the data well. When investigating how quickly bats change pulse characteristics, several models were compared based on their AIC values, and the model with the zero-truncated Poisson family was found to fit the data best.

Overall, the models fit the data well and explained more variance than their respective null models (Table S2). Furthermore, all interactive effects in the four models were significant (Table S3).