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RESEARCH ARTICLE

The frugivorous bat *Carollia perspicillata* dynamically changes echolocation parameters in response to acoustic playback

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ABSTRACT

Animals extract behaviorally relevant signals from 'noisy' environments. Echolocation behavior provides a rich system testbed for investigating signal extraction. When echolocating in acoustically enriched environments, bats show many adaptations that are believed to facilitate signal extraction. Most studies to date focused on describing adaptations in insectivorous bats while frugivorous bats have rarely been tested. Here, we characterize how the frugivorous bat *Carollia perspicillata* adapts its echolocation behavior in response to acoustic playback. Since bats not only adapt their echolocation calls in response to acoustic interference but also with respect to target distances, we swung bats on a pendulum to control for distance-dependent call changes. Forward swings evoked consistent echolocation behavior similar to approach flights. By comparing the echolocation behavior recorded in the presence and absence of acoustic playback, we could precisely define the influence of the acoustic context on the bats' vocal behavior. Our results show that *C. perspicillata* decrease the terminal peak frequencies of their calls when echolocating in the presence of acoustic playback. When considering the results at an individual level, it became clear that each bat dynamically adjusts different echolocation parameters across and even within experimental days. Utilizing such dynamics, bats create unique echolocation streams that could facilitate signal extraction in noisy environments.

KEY WORDS: Echolocation, Active sensing, Bioacoustics, Signal interference, Spatial orientation, Jamming avoidance response

INTRODUCTION

For orientation, echolocating bats emit biosonar calls and listen to echoes arising from reflections off surrounding objects (Kössl et al., 2014; Moss and Surlykke, 2010; Simmons, 2012). Spectro-temporal parameters of echoes inform the animals about the position and identity of objects nearby (Wohlgemuth et al., 2016b). To gain spatial information, bats must assign incoming echoes to their corresponding calls (Corcoran and Moss, 2017; Suga et al., 1983; Ulanovsky et al., 2004). Call-echo assignments become challenging, however, when biosonar signals from conspecifics overlap (Corcoran and Moss, 2017; Levin et al., 2013; Parsons et al., 2003; Ulanovsky and Moss, 2008). Under these circumstances, bats show a large repertoire of echolocation adjustments that are thought

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to improve signal extraction (for review, see Jones and Conner, 2019). These adjustments include shifting call spectra to reduce signal overlap, increasing call level to improve the signal-to-noise ratio, adapting call duration to reduce temporal overlap between signal and noise, and increasing the redundancy of echo information by emitting groups of calls (call strobes; Roverud and Grinnell, 1985a,b). Some species reduce their call rate to minimize the risk of acoustic interference (Adams et al., 2017; Jarvis et al., 2013; Obrist, 1995). Our current understanding of why bats show such a large variety of echolocation adjustments is sparse. It remains speculative whether the adjustments observed are species specific or even individual specific and/or depend on the environmental context in which bats vocalize (e.g. the distance between the bat and obstacles or the clutter density; Jones and Conner, 2019). Alternatively, echolocation adjustments could be used in combination to ensure signal discriminability (Amichai et al., 2015; Hage et al., 2013; Tressler and Smotherman, 2009). This hypothesis is supported by data from the frugivorous bat *Phyllostomus discolor* (Luo et al., 2015), which increases call intensity and call duration in response to acoustic interference. The abovementioned echolocation adjustments do not covary, meaning that intensity and duration are separately controlled by the animal. Although *P. discolor* demonstrated a slight decrease in call bandwidth when dealing with noise, spectral adjustments in response to acoustic interference have not been analyzed in detail for frugivorous bats thus far. In this study, we characterized, for the first time, echolocation adjustments in the echolocation behavior of the frugivorous bat *Carollia perspicillata* when they echolocated in the presence of acoustic interference. *C. perspicillata* orients in highly cluttered environments (Thies et al., 1998); environmental conditions in which call echo assignment must be precise to avoid collisions with surrounding obstacles. Since *C. perspicillata* and *P. discolor* are both frugivorous and share habitats, we reasoned that – like *P. discolor* – *C. perspicillata* could also rely on different call adjustments to overcome acoustic interference. To deepen the understanding of echolocation behavior shown by frugivorous bats under 'noisy' environments, we also tested if individual bats rely on different echolocation adjustments and if they can switch between the adjusted parameters at any timepoint during echolocation. Individual bats were restrained on the mass of a swinging pendulum (Fig. 1A). The pendulum offers a behavioral paradigm whereby bats can actively echolocate in controlled scenarios, and can be replicated over several trials (Beetz et al., 2016, 2017; Henson et al., 1982; Macias et al., 2016). In our test trials, during forward swings – which mimicked a bat closing in on a target – the animals were acoustically stimulated with patterned echolocation calls broadcasted from a speaker, which traveled with and pointed towards the animal. The call design and emission patterns recorded during test trials were then compared with those recorded during control trials in which bats were swung in the absence of playback stimuli. During test trials, we examined whether bats would change
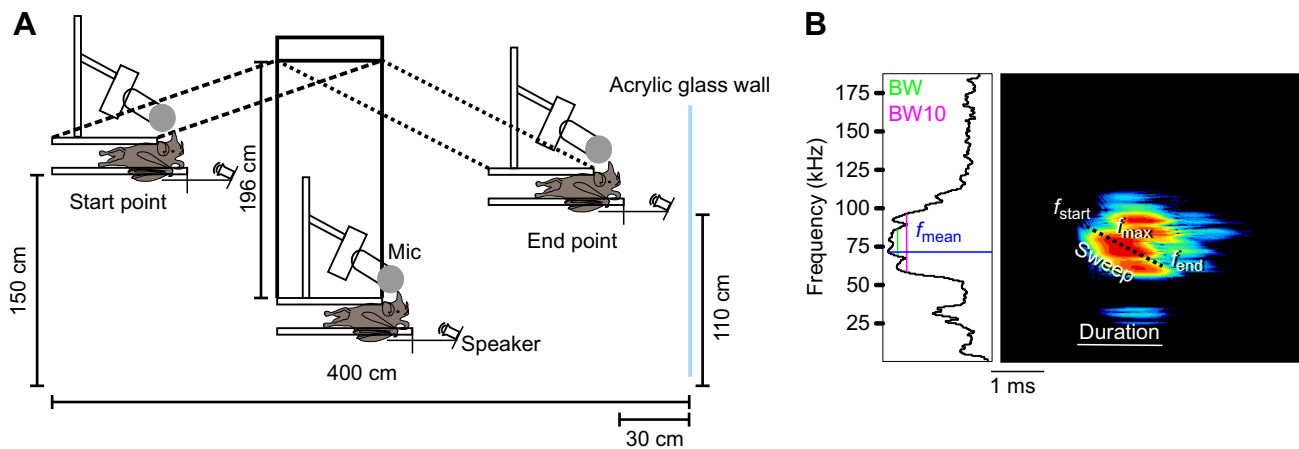
Fig. 1. Behavioral paradigm and representative echolocation call. (A) Schematic side view of the pendulum. The bat was positioned on a pendulum and swung towards an acrylic glass wall. During the swing, the bat emitted echolocation calls that were recorded together with the echoes by an ultrasound microphone. For test trials, the bat was stimulated with playback stimuli that were composed of a previously recorded echolocation call of the tested bat. The playback stimuli were emitted with a speaker pointing towards the bat's head. Microphone and speaker traveled with the bat. (B) Power spectrum (left) and spectrogram (right) of a representative echolocation call recorded with the pendulum. Different call parameters were measured to characterize spectro-temporal call properties. Spectral parameters that were measured included initial (f_{start}), center (f_{center}), terminal (f_{end}), mean (f_{mean}), maximum amplitude (f_{max}) peak frequency and bandwidths at five (BW5) and ten dB (BW10) below the f_{mean}. Call duration represents one of the temporal echolocation parameters that was considered in the analysis. The sweep rate represents the difference of f_{end} and f_{start} (f_{end}–f_{start}) divided by the call duration.

different echolocation parameters, including call duration, call level, call frequency composition and call emission pattern. The results obtained indicate that *C. perspicillata* indeed use a rich, dynamic toolkit of call adjustments when echolocating in noisy environments.

MATERIALS AND METHODS
Animals
Experiments were conducted on 32 adult bats (15 females and 17 males) of the species *Carollia perspicillata* (Linnaeus 1758). The bats were bred and housed in a colony at the Institute for Cell Biology and Neuroscience (Goethe University, Frankfurt). The experiments complied with all current German laws on animal experimentation and in accordance with the Declaration of Helsinki. All experimental protocols were approved by the Regierungspräsidium Darmstadt (experimental permit #FU-1126).

Pendulum paradigm and audio recordings
For controlling the behavioral context, bats were restrained between an acrylic plate and a platform on the mass of a pendulum. The acrylic plate was carefully attached to the platform and the bat was positioned in the space between the plate and the platform. Then, the pendulum was hand-released so that the bat swung towards an acrylic glass wall (50×150 cm, Fig. 1A) (Beetz et al., 2016, 2017; Henson et al., 1982; Macias et al., 2016). During the forward swing, which took an average of 1.5 s, the bats emitted echolocation sequences that were recorded, together with their echoes, by an ultrasound sensitive microphone (CM16/CMPA, Avisoft Bioacoustics, Germany). The microphone had a sensitivity of 50 mV Pa⁻¹ and an input-referred self-noise level of 18 dB sound pressure level (SPL), as reported by the manufacturer. The frequency response curve was flat (±3 dB, as specified by the manufacturer) in the range from 30–130 kHz. The microphone traveled with the mass of the pendulum and was medially positioned above the bat's head. The membrane of the microphone was adjusted to be as close as possible to the bat's ears (~4 cm). The microphone was connected to a sound acquisition system (Ultra Sound Gate 116Hm mobile recording interface, +Recorder Software, Avisoft Bioacoustics,

Germany). After one forward and one backward swing, the pendulum was manually stopped by the experimenter. All experiments were performed in darkness with the eyes of the bats covered with Bepanthen cream (Bayer Vital GmbH) and a small piece of aluminium foil attached on top of the cream. This prevented the bats from relying on visual rather than acoustic information during the swing. Vocalizations emitted by bats during the swing could clearly be classified as echolocation calls and were distinguishable from distress calls (Hechavarría et al., 2016) which were not recorded during any pendulum swing. All tested bats were naïve because individuals rapidly habituate to the pendulum after a few swings and stop emitting calls (these habituations become clear when comparing the number of calls emitted across successive days, as shown in the present study).

Call variability across trials
Nine bats (five females, four males) were swung two to four times (two trials in two bats, three trials in six bats, and four trials in one bat) in a row, to investigate the call variability across trials. These nine bats were also tested in the presence of playback stimuli. During each swing, the bats emitted 16.65±5.04 calls (mean±s.d.).

Call variability across different individuals from a colony
To gain an overview of the call variability across individuals, 32 adult bats (15 females and 17 males) were swung in the pendulum. Each bat was tested once to minimize habituation effects, which is represented by a reduction of emitted calls over subsequent swings. During each swing, the bats emitted 19.02±10.36 calls (mean±s.d.). For quantification, we computed a similarity index for each pair of bats. This index represents the proportion of tested call parameters that are significantly not different between the pair of bats ($P \geq 0.05$; non-parametric Kruskal–Wallis tests and Tukey–Kramer multiple comparison *post hoc* tests). An index of 1 means that we could not find any call parameter that was different between the pair of bats.

Call variability across days
Ten bats (5 females and 5 males) were tested in the pendulum over two (N=3) to three (N=7) consecutive days. These 10 bats were

also in the presence of playback stimuli and nine of these bats were also tested in the control experiment examining the call variability across trials. During the swing, the bats emitted 16.18±3.99 calls (mean±s.d.).

Influence of acoustic playback on echolocation behavior

To test the effect of acoustic interference on echolocation behavior, 10 bats (5 females and 5 males) were swung in the pendulum while they were acoustically stimulated with a playback stimulus (see below). We compared the echolocation behavior recorded in the absence of playback stimuli (control trials) with the one shown in the presence of playback (test trials). Our reasoning was that because the behavioral context was invariant during control and test trials, except for the occurrence of the playback stimulus, we could correlate adaptations in the echolocation behavior with the presence/ absence of the playback.

Bats were tested in one control trial followed by test trials, in which an echolocation call recorded during the forward swing of the control trial was randomly selected to construct an individual-specific playback stimulus. To minimize habituation to the pendulum paradigm, we decided to have only one control trial per session (per animal and day). Call parameters from subsequent control trials do not vary across swings (except for call intervals, Table S2). Thus, one control trial is enough to characterize a bat's echolocation behavior in the absence of playback stimuli. After the control trial, the playback stimulus was constructed, which took 2–5 min. During that time, the bat was kept restrained while the pendulum was still. The playback stimulus consisted of an echolocation call that was presented as quartets with a call interval of 25 ms and the quartets were repeated with an inter-quartet interval between 130 and 150 ms. The intensity of the playback stimulus was adjusted to rms values (of single calls) between 80 and 90 dB SPL for all animals. An overview of the call parameters used for constructing playback stimuli is shown in Table S1. We reasoned that using an echolocation call of the tested animal, as a playback stimulus, would be the most effective way of achieving acoustic jamming. This is supported by the fact that inter-individual differences in call design can be detected by bats, which reduces signal interference (Amichai et al., 2015; Masters et al., 1991; Yovel et al., 2009). During test trials, the playback stimulus was presented from an ultrasound speaker (MK 103.1 Microtech Gefell Microphone Capsule used as speaker) that was flat in the range from 5 to 120 kHz (mean±s.d. level in calibration curve 84±3 dB SPL; the speaker's protection cap was replaced with a self-made cap to prevent energy loss at high frequencies). The speaker was placed pointing towards the bat's head at a distance of 20 cm. The relatively short distance between speaker and animal and the bat's restrained head prevented situations in which the bat could reduce acoustic interference via motor responses such as head 'waggling' (Wohlgemuth et al., 2016a). Thus, the bats had to rely mostly on changes in call design or emission pattern to minimize signal interference. For the test session, each bat was swung in the presence of playback for several times until the bat emitted less than seven calls per trial. If the latter occurred, the bat was released from the pendulum and returned to the colony. Eight (4 from each sex) out of 10 bats were tested at the following day. For each day we recorded an initial control trial to construct a day-specific, playback stimuli. This controlled for changes of the call design that may occur across days and that might bias our analysis. On average, 3.6±1.5 test trials (means± s.d.) were recorded from each bat per day. During the swing, the bats emitted 15±5.72 calls (means±s.d.).

Echolocation parameters

Since the time pattern of the playback stimuli was kept constant, we could discriminate between biosonar signals emitted by the bat and the playback stimuli. The call emissions were manually tagged in the software Avisoft SAS Lab Pro (Avisoft Bioacoustics, Germany). To characterize the echolocation calls, different call parameters were measured in Avisoft SAS Lab Pro. The present study focused on call level, call duration, peak frequency at different call time points (start, end, maximum amplitude, and mean), bandwidth 5 (BW5), bandwidth 10 (BW10) and sweep rate (Fig. 1B). Peak frequencies (frequencies with the maximum energy at particular time points of the call or on average of a call) are likely to be the most salient spectral information of the echo that would suffer least from reflective attenuation. BW5 and BW10 represent frequency ranges at 5 and 10 dB below the mean peak frequency (Fig. 1B). The sweep rate was calculated by subtracting the initial peak frequency from the terminal peak frequency and dividing that number by the call duration.

The call emission pattern was characterized by measuring the call intervals and analyzing call groups. Analysis of the call groups was conducted using custom-written scripts in MATLAB 2014 (MathWorks, USA). Call groups were defined according to the following two criteria (Beetz et al., 2018; Kothari et al., 2014). First, the 'island' criterion defined call groups that were isolated in time. 'Isolation' was fulfilled as soon as the preceding and following call intervals of a call group were 20% longer than the call intervals within call groups. If the island criterion was fulfilled, the second criterion, the so-called 'stability' criterion, defined the size of the call groups indicated by the number of calls belonging to a group. The stability criterion was fulfilled if the call intervals within call groups were stable with a 5% tolerance. Next, we calculated a strobe index for each animal and each condition (control and test trial). The strobe index represented the relative number of calls that were emitted as groups.

Statistics

For statistical analysis, we used GraphPad Prism 7 (GraphPad Software, USA; $*P<0.05$; $**P<0.005$; $***P<0.001$; $****P<0.0001$). For analyzing distance-dependent changes of the echolocation behavior in the pendulum, non-parametric Kruskal–Wallis tests and Dunn's multiple comparison *post hoc* tests were computed. For analyzing individual specific call adaptations in response to acoustic playback, control and test trials were directly compared from each animal by performing non-parametric Mann–Whitney (in case of non-Gaussian distribution according to D'Agostino and Pearson normality test; alpha=0.05) or parametric *t*-tests (in case of Gaussian distribution according to D'Agostino and Pearson normality test; alpha=0.05). For a comparison of the echolocation behavior between subsequent trials, non-parametric Kruskal–Wallis tests and Dunn's multiple comparison *post hoc* tests (in case of non-Gaussian distribution according to D'Agostino and Pearson normality test; alpha=0.05) or ordinary one-way ANOVA and Tukey's multiple comparison *post hoc* tests (in case of Gaussian distribution according to D'Agostino and Pearson normality test; alpha=0.05) were computed.

RESULTS

Pendulum paradigm mimics a natural approach flight

Bats swinging on a pendulum often emit echolocation calls (Henson et al., 1982; Macias et al., 2016). Since a pendulum allows behavioral context repetition, it allows us to describe echolocation behavior under controlled conditions. This is

important for testing the influence of acoustic playbacks on echolocation behavior, independent from changes in the echolocation behavior linked to target distances.

First, we quantified if pendulum forward swings evoked consistent distance-dependent adjustments of the echolocation behavior in *C. perspicillata*. Based on 32 forward swings, each recorded from a different individual, we found that the bats shortened their call duration and inter-call intervals with decreasing target distance (Fig. 2A,B). In addition, with decreasing target distance, the bats increased their call intensity, starting peak frequency and peak frequency at the call's maximum energy (peak frequency max.; Fig. 2C–E). Since the distance-dependent adjustments in call duration and call interval are comparable in the pendulum (laboratory condition) and in free-flying bats (Thies et al., 1998), we concluded that a forward swing

in the pendulum mimics a bat zooming in on a target in natural conditions.

Next, we tested the inter-swing variability in the echolocation behavior of 9 bats (5 females, 4 males, Table S2). Call duration, intensity, starting peak frequency, sweep rate, terminal peak frequency, peak frequency at call's maximum energy, mean peak frequency, BW5 and BW10 did not vary over subsequent swings (blue cells in Table S2; $P>0.05$; Mann–Whitney test for females F9 and F10; Kruskal–Wallis test for F8, F11, F12, males M9, M10, M11, M12) indicating that one forward swing reliably represents the echolocation behavior of an individual bat. Only four animals (F9, F10, F11 and M12) increased their call intervals across subsequent trials (Mann–Whitney test for F9 and F10; Kruskal–Wallis test for F11 and M12; $P<0.005$), which may indicate that the bats habituated to the pendulum and therefore decreased calling rate.
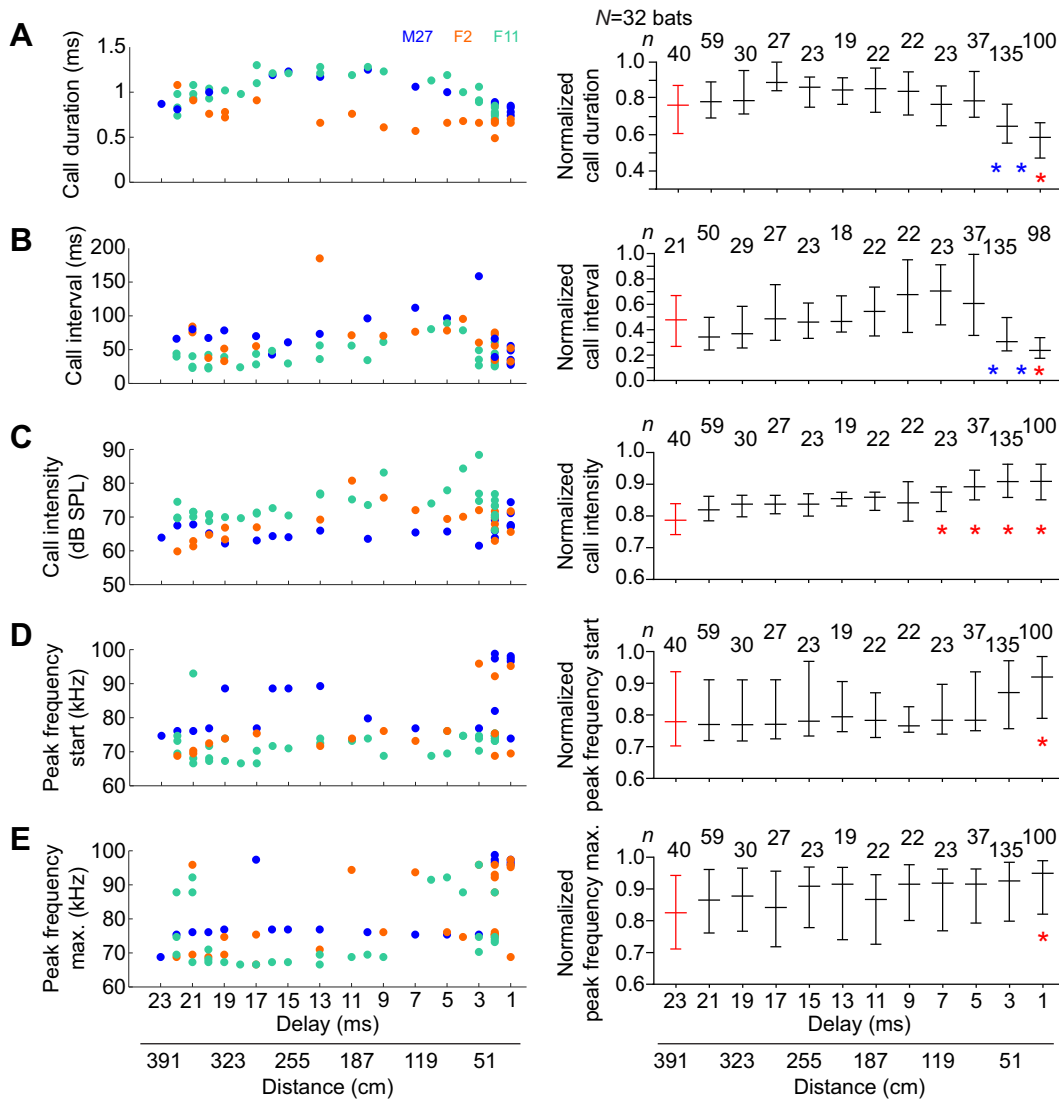

Fig. 2. Distance-dependent changes of echolocation parameters during forward swings of the frugivorous bat *Carollia perspicillata*. *C. perspicillata* reduces call durations (A) and call intervals (B) with shorter object distances. Call intensities (C), initial peak frequency (D) and maximum peak frequency (E) slightly increase with shorter object distances. Subfigures on the left represent examples from three bats (M27, F2, F11). Each dot denotes a call. Panel on the right represents the median and the interquartile range of data from 32 bats in which each value was normalized to the maximum value of the corresponding trial. Blue stars indicate significant differences ($P<0.05$) between subsequent echolocation calls. Red stars indicate significant differences ($P<0.05$) with calls emitted at echo delays between 23 and 22 ms (red data point). Data points without blue or red stars are not significantly different from the preceding or the first data point (red data point), respectively. Kruskal–Wallis test and Dunn's multiple comparison *post hoc* test.

Call variability across individual bats

Before testing the influence of acoustic playback on the echolocation behavior of *C. perspicillata*, we wanted to quantify the potential of acoustic interference when encountering conspecifics. As the risk of acoustic interference increases, the more similar echolocation calls are across individuals. To compare echolocation calls across individuals, we tested 32 bats in our pendulum paradigm and compared the calls across individuals. We statistically (non-parametric Kruskal–Wallis tests and Tukey–Kramer multiple comparison *post hoc* tests) compared call level, call duration, peak frequency at different call time points (start, end, maximum amplitude, and mean), BW5, BW10 and sweep rate across each bat and calculated a similarity index between each pair of bats (496 possible pairs). A similarity index of 1 means that no call parameters tested differ between bats in a pair ($P \geq 0.05$; non-parametric Kruskal–Wallis tests and Tukey–Kramer multiple comparison *post hoc* tests). Similarity indices from each pair were plotted as heatmaps (Fig. 3A). When comparing the similarity indices across all pairs (Fig. 3B), it becomes clear that echolocation calls from most bats do not differ in any call parameter analyzed in the present study (mean±s.d. of similarity index=0.85±0.15). Thus, acoustic interference may be a critical problem with which *C. perspicillata* may have to cope.

Call adjustments in response to acoustic playback

When bats (N=10 for 2 consecutive days resulting in n=20 values; one value per animal and per day) were swung in the presence of the playback stimulus, they significantly decreased the terminal peak frequency (P=0.03; paired *t*-test; Fig. 4D). According to the remaining call parameters, the responses of the bats were quite variable (Fig. 4) and there was no common adjustment that all bats showed. Next, we tested the hypothesis that each bat could focus on different echolocation parameters for coping with acoustic interference. Hereby, we determined, within each animal, which call parameters are adjusted in response to the playback stimulus (Table 1).

Four bats (F11, F12, M9, M12) increased the tendency of grouping their calls into call packs (exemplarily shown for F11 in Fig. 5A; test trial, see also population data in Fig. 5E). Six bats

(F8, F10, F11, M10, M12 and M13) varied their call intervals. However, with respect to changes in call intervals, only a reduction (observed in 2 bats, F11, M13) could be interpreted as a response to the playback stimulus. We reasoned that increased call intervals may also reflect habituation to the pendulum paradigm (see also Table S2). Three bats (F8, M9, M13) increased and another 3 bats decreased (F9, M11, M12) call intensity during the test trials (Table 1; example in Fig. 5B). Five bats changed their call duration, 2 shortened (F8, M11), 2 lengthened (M9, M10) and 1 shortened their 'short delay calls' and lengthened their 'long delay calls' (F9, Fig. 5B; Table 1). The adjustments in call duration of F9 indicated that some bats differentially adapt 'long delay calls' (delay >6 ms) and 'short delay calls' (delay ≤6 ms) in response to playbacks. Changes in call spectra were sometimes prominent (Fig. 5C,D) but also varied across animals (Table 1). Calls shown in Fig. 5C,D were recorded as the bat had approximately the same distance to the target (~2 m). Seven out of 8 bats that changed their calls' sweep rate decreased it when in the presence of playback. This indicated that the call frequency changed more slowly during the test compared to the control trials. Changes in the sweep rate could be caused by changes of the call's frequency range or by changes in call duration. Because lowering the sweep rate was not associated with lengthening the call, the sweep rate was mainly affected by changes in the frequency range. Seven out of 10 animals changed either the BW5 or BW10 of the calls in the test trials. These changes could either be a BW decrease (shown by 40% of the bats tested; F8, F10, M11, M13) or an increase (shown by 30%; F9, F12, M9). Detailed data from 3 animals (A: F8; B: F9; C: M9) are plotted as boxplots in Fig. 6A–C. For reasons of visualization, only call parameters that differed between the test and the control trials are plotted. Data from the remaining animals are presented in Fig. S1. In conclusion, each bat adjusted at least one call parameter in response to the playback stimuli. M11 was the only individual that did not change its call design (short delay calls) during the test trials. Overall, the bats changed different combinations of their call parameters, indicating that there was no common rule as to how to adapt to the playback stimuli.
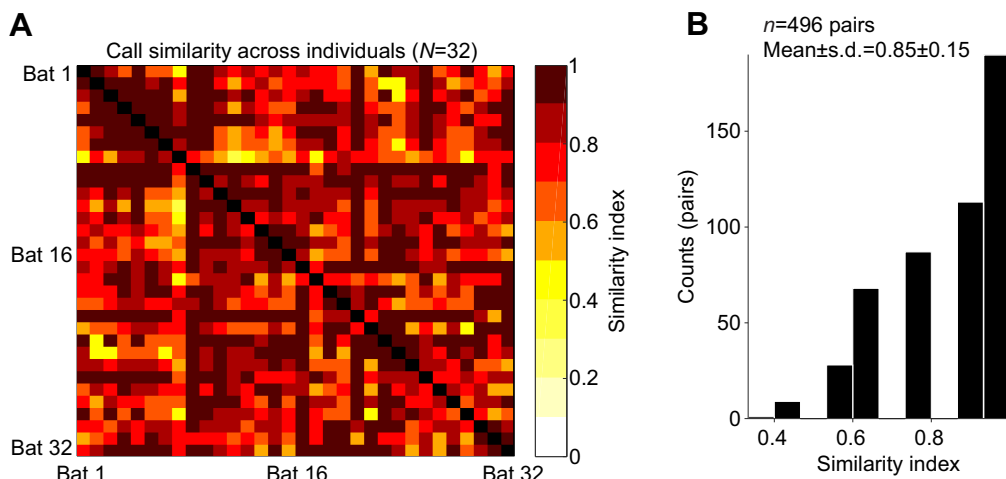

Fig. 3. Comparison of call design across 32 bats. (A) Heatmap represents the similarity of echolocation calls across different pairs of bats (n=496 different pairs for N=32 animals). The similarity index varies between 0 and 1. An index of 0 means that all tested call parameters (a total of nine) were significantly different between the compared animals (P<0.05) while an index of 1 means that no call parameter was significantly different between the corresponding pair ($P \geq 0.05$; non-parametric Kruskal–Wallis tests and Tukey–Kramer multiple comparison *post hoc* tests). (B) Histogram summarizing the similarity indices from 496 pairs. The mean similarity index and its standard deviation are denoted on top of the histogram.
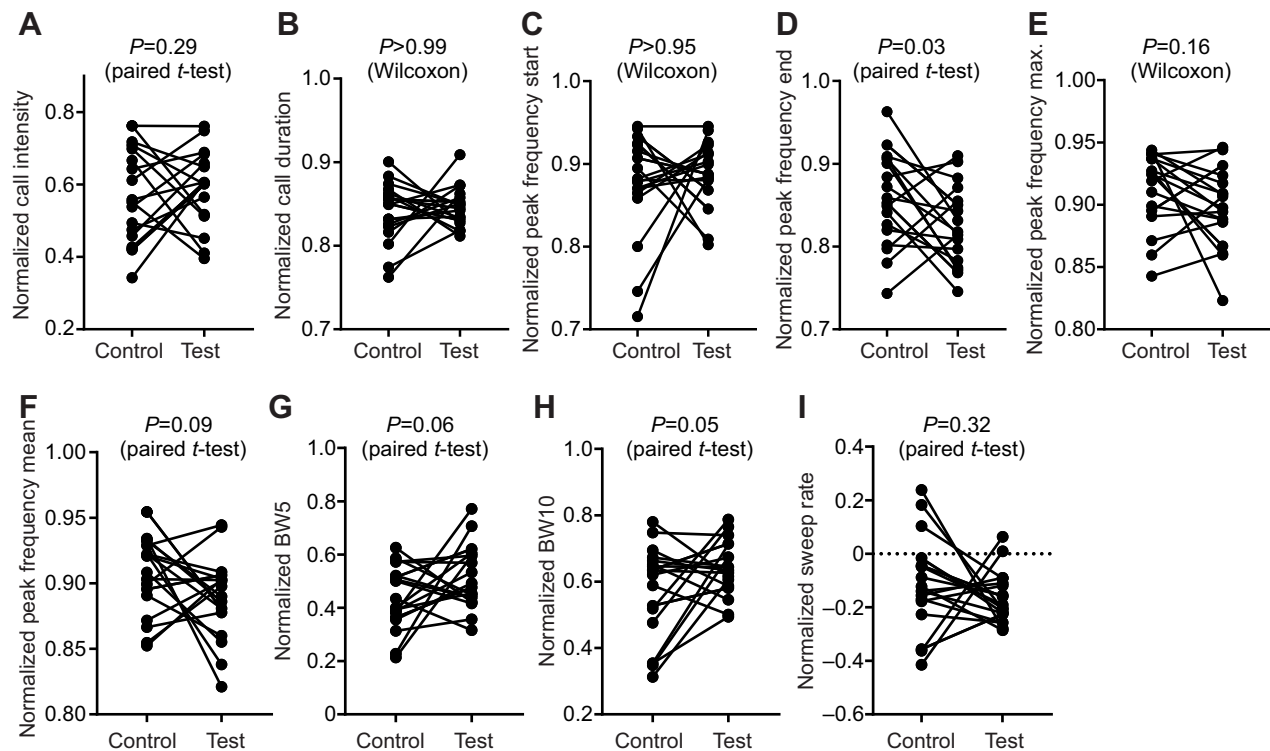
Fig. 4. Call adjustments in response to acoustic playback. (A–I) Before and after scatter plots demonstrate adjustments of different call parameters: (A) call intensity; (B) call duration; (C) peak frequency start; (D) peak frequency end; (E) peak frequency at maximum call amplitude; (F) peak frequency mean; (G) bandwidth 5; (H) bandwidth 10; (I) call sweep rate of *C. perspicillata* (*n*=20 values; *N*=10 bats tested for two consecutive days) in response to acoustic playback. Bats responded quite variably to the playback. Only the terminal peak frequency (D) significantly decreased in the presence of playback (*P*=0.03; paired *t*-test). In cases of normalized distributions, a parametric paired *t*-test was conducted. Otherwise, a non-parametric Wilcoxon signed rank test was performed.

Bats dynamically change different echolocation parameters across trials and days

We were interested in assessing if each bat shows the same echolocation adjustments in response to the playback or whether the adjusted parameters change across days or even across trials on the same day. However, before characterizing the temporal dynamics of the adjustments, we quantified the variability of the call design across subsequent days under controlled conditions (absence of playback stimulus). We tested 10 bats in the absence of playback stimuli for 2 (F1, M3, M7) or 3 (F2, F3, F5, F7, M1, M4, M6) subsequent days (Table S3). Although the bats did not vary their call design across subsequent control trials on the same day (Table S2), they dramatically varied their call design across subsequent days (Table S3). Thus, to test if bats change their adjustments in response to the playback stimulus across days, we recorded an initial control trial on each day and compared the echolocation behavior from the day-specific control trial with the one recorded during test trials. Moreover, to perform a trial-by-trial analysis and to gather enough data points for statistical analysis, we pooled data from long and short delay calls. During the test trials, bats emitted slightly fewer calls than during control trials (median *n* of calls: 16.5 control and 13 test; Mann–Whitney test: *P*=0.036). When the call parameters from F9 across days were compared (Fig. 7; Table S4), it became clear that the adjustments of call duration (Fig. 7C), starting (Fig. 7B), maximum (Fig. 7F), and mean peak frequency (Fig. 7H), BW5 (Fig. 7G) and BW10 (Fig. 7I) exclusively occurred on day 1. On day 2, bat F9 mainly changed call intensity (Fig. 7A), terminal peak frequency

Table 1. Changes in call parameters induced by the presence of playback stimuli

	F8		F9		F10		F11		F12		M9		M10		M11		M12		M13	
Delay	l	s	l	s	l	s	l	s	l	s	l	s	l	s	l	s	l	s	l	s
Interval	++	+++			+	++	– –						++	+			+			– –
Duration	–		+++	– – –								+	+		– –					
Intensity		+++		– – –							++				– –		– – –	– – –		++
PF start			–	– – –					+											
Sweep rate		– – –	– – –	– – –		–		–		+		– –					–			
PF end		+++			+								– – –	–	– – –	– –		– –		
PF max.			– – –	– –	– –				–				– –					++		
PF mean			– – –	– –			– –													
BW5	– – –			++								+								
BW10	– – –			+++		– – –			++			+				– –				–

+/–, higher/lower values for test than for control trials (+ and –, *P*<0.05; ++ and – –, *P*<0.01; +++ and – – –, *P*<0.001); F, female; M, male; l, long delay calls; PF, peak frequency; s, short delay calls.
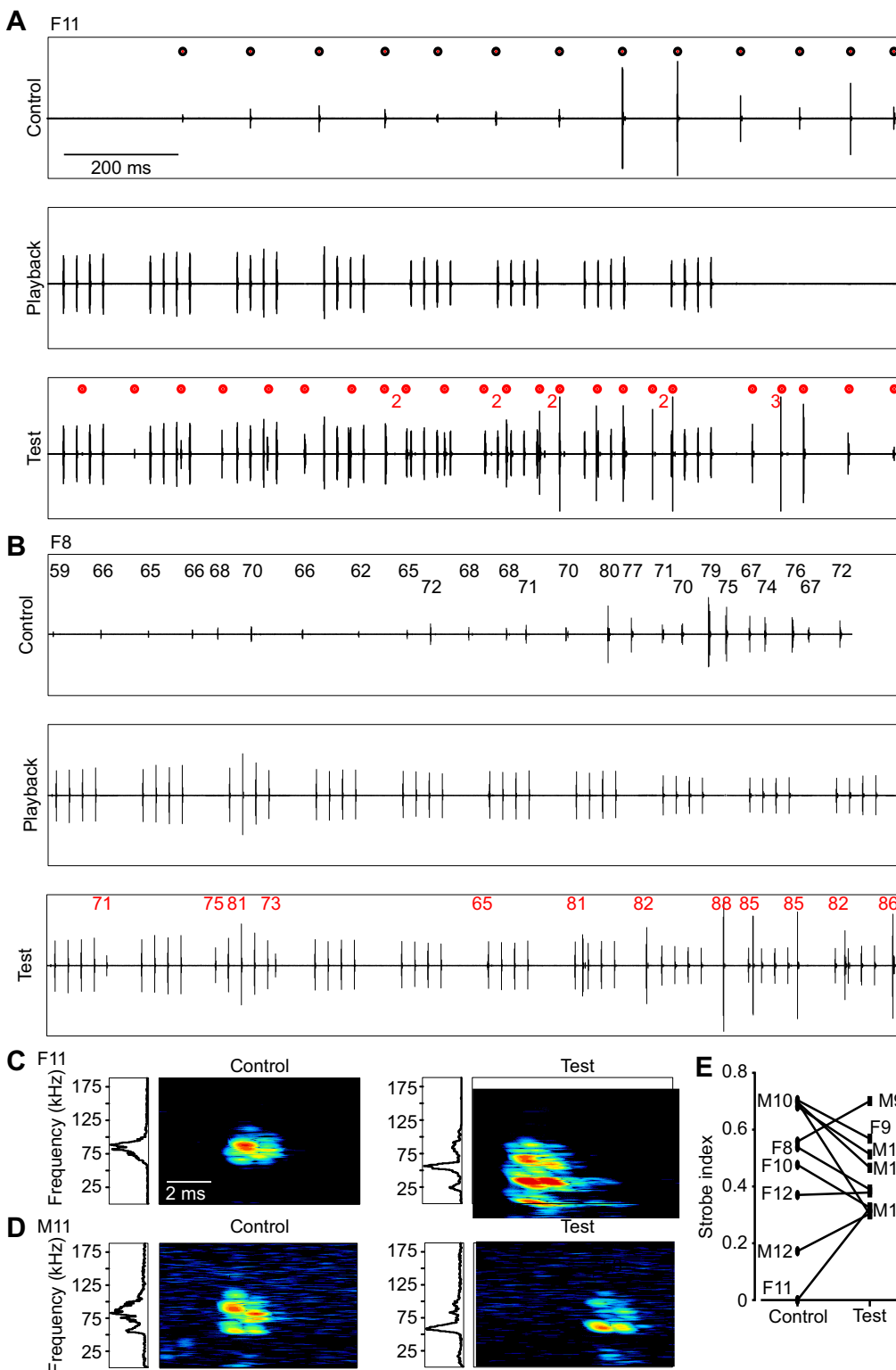
Fig. 5. Examples of echolocation adjustments in response to playback stimuli. (A) Oscillograms of one control trial (top), the playback stimulus (middle), and one test trial (bottom) from female 11 (F11). Time points of call emissions are indicated by black or red dots above each oscillogram. During the control trial, the bat did not emit echolocation call groups. During the test trial, the bat grouped some calls into doublets (indicated by a '2') or triplets (indicated by a '3'). Note that the jamming stimulus was recorded in addition to the echolocation calls of the test trial. Thus, oscillogram deflections without a dot represent signals coming from the playback stimulus. (B) Oscillogram of one control trial (top), the playback stimulus (middle) and one test trial (bottom) from female 8 (F8). In comparison to the calls emitted during the control trial, the call intensity was increased during the test trial. Numbers above each emitted call indicate the call intensity (dB SPL). (C,D) Power spectra (left) and spectrograms (right) of representative calls emitted during the control and test trial for two individuals (F11, M11). To exclude distance-dependent changes in the call design, all four calls were recorded as the bat was ~2 m away from the acrylic wall. Both bats decreased the bandwidth and mean peak frequency of their calls during the test trials as compared with the calls recorded during the control trials. (E) Tendency of emitting grouped calls (strobe index) under control and test conditions in all bats tested (N=10).

(Fig. 7D), and sweep rate (Fig. 7J). As already mentioned, increments in call interval (Fig. 7E) did not necessarily represent a response to our playback; possibly, they represented habituation to the paradigm across trials/days.

We observed that echolocation adjustments not only varied across days, but also across subsequent trials (Table S4). For example, F9 changed the calls' mean peak frequency in three (trial 3, 4, 5) out of five trials at day 1 (Fig. 7H). Changes of other call parameters varied less dramatically across trials of the same day. In all trials on day 1, F9 decreased its starting (Fig. 7B) and maximum peak frequency (Fig. 7F). For detailed data from the remaining nine animals see Table S4. Overall, we found in 56 out of 67 test trials (83.6%) statistically significant differences between the control and test trials (Table S4). In 11 test trials, the bats did not change any call parameters compared with the control trial.
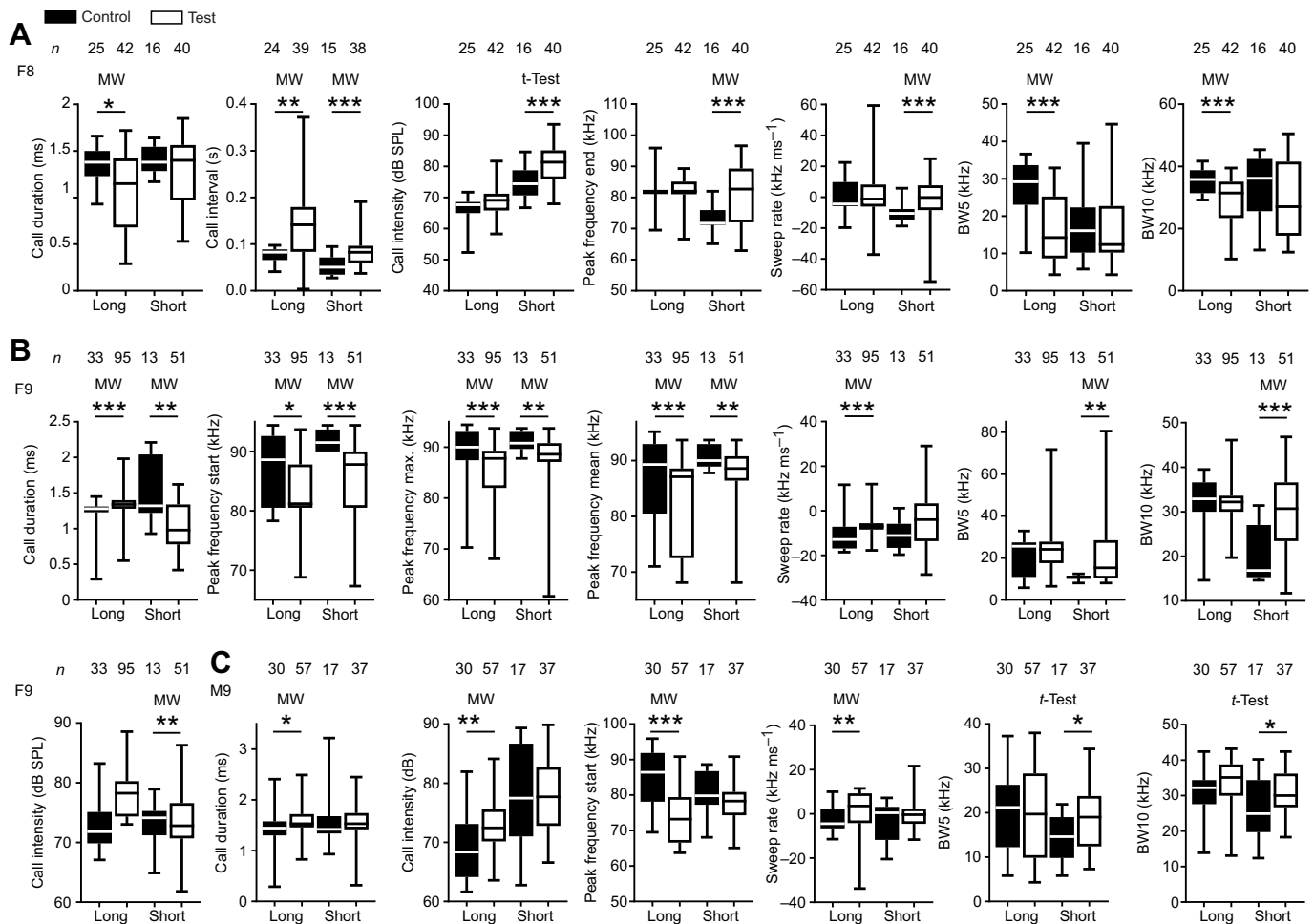
Fig. 6. Individual specific call adjustments in response to playback stimuli. (A–C) Boxplots (whiskers represent minima and maxima, bars are medians, and boxes and whiskers represent interquartile range and minimum/maximum values, respectively) from three individuals: (A) female 8 (F8), (B) female 9 (F9), (C) male 9 (M9), showing call parameters that bats changed in response to the playback stimulus. Data obtained in absence and presence of playback stimulus are indicated by black and white boxplots, respectively. Echolocation calls that are followed by an echo within 6 ms were grouped into 'short delay calls'. Echoes following a call by more than 6 ms were grouped into 'long delay calls'. Note that each bat adjusted different call parameters under test conditions. The amount of analyzed calls for the corresponding parameter are indicated at the top of each boxplot. MW, Mann–Whitney test; *P<0.05; **P<0.005; ***P<0.001.

Bats dynamically adjust different echolocation parameters within trials

What could have happened during test trials when we did not find an echolocation adjustment? For these trials, was the acoustic interference too weak to evoke adjustments? Alternatively, could the bats have dynamically changed their adjustments during trials, so that the adjustments would not be detectable when pooling calls from an entire swing? To assess the latter idea, we compared parameters of each call from the test trial with the same parameters in the call used to construct the playback stimulus. The upper color maps, in Fig. 8A,B, exemplarily show the relative differences between call parameters and the playback parameters for two trials in two different bats (M9 and F12). The calls are ordered along columns in which the leftmost column represents the call with the longest echo-delay and the rightmost column represents the call with the shortest echo delay. Each line represents the relative difference of a call parameter with respect to a playback parameter. This result was calculated by subtracting the playback parameters from the call parameters and by normalizing the difference against its absolute maximal difference for the entire trial. The darker the red and blue patches are, the more positive and negative were the call parameters in comparison to the playback stimulus. Based on the trial in

Fig. 8A, the bat initially emitted calls with lower starting peak frequencies (peak start) and call intensities than the playback stimulus. At an echo delay of ~3 ms (between the 12th and 13th call, white dashed line in Fig. 8A), the bat abruptly switched the adjusted echolocation parameters and increased the maximum and mean peak frequency while decreasing the bandwidth of subsequent calls. To better visualize abrupt adjustments, we calculated the differences of the parameters of subsequent calls and plotted the values in the bottom color maps shown in Fig. 8A,B. We defined an abrupt adjustment when the considered parameter varied by more than 50% between subsequent calls. For example, according to Fig. 8A, the terminal (peak end), maximum peak frequency (peak max.), and sweep rate of call 13, are more than 50% higher than the ones of call 12. This outcome is indicated by red cells at the corresponding column (white dashed line) in the lower color map of Fig. 8A.

Abrupt call adjustments were also apparent in other trials, like the one presented in Fig. 8B. Here, they mainly occurred at around 2.5 ms echo delay (white dashed line) by decreasing the call intensity, starting (peak start), and terminal frequency (peak end) while the maximum peak frequency (peak max.) as well as the call bandwidths (BW5 and BW10) abruptly increased. By considering
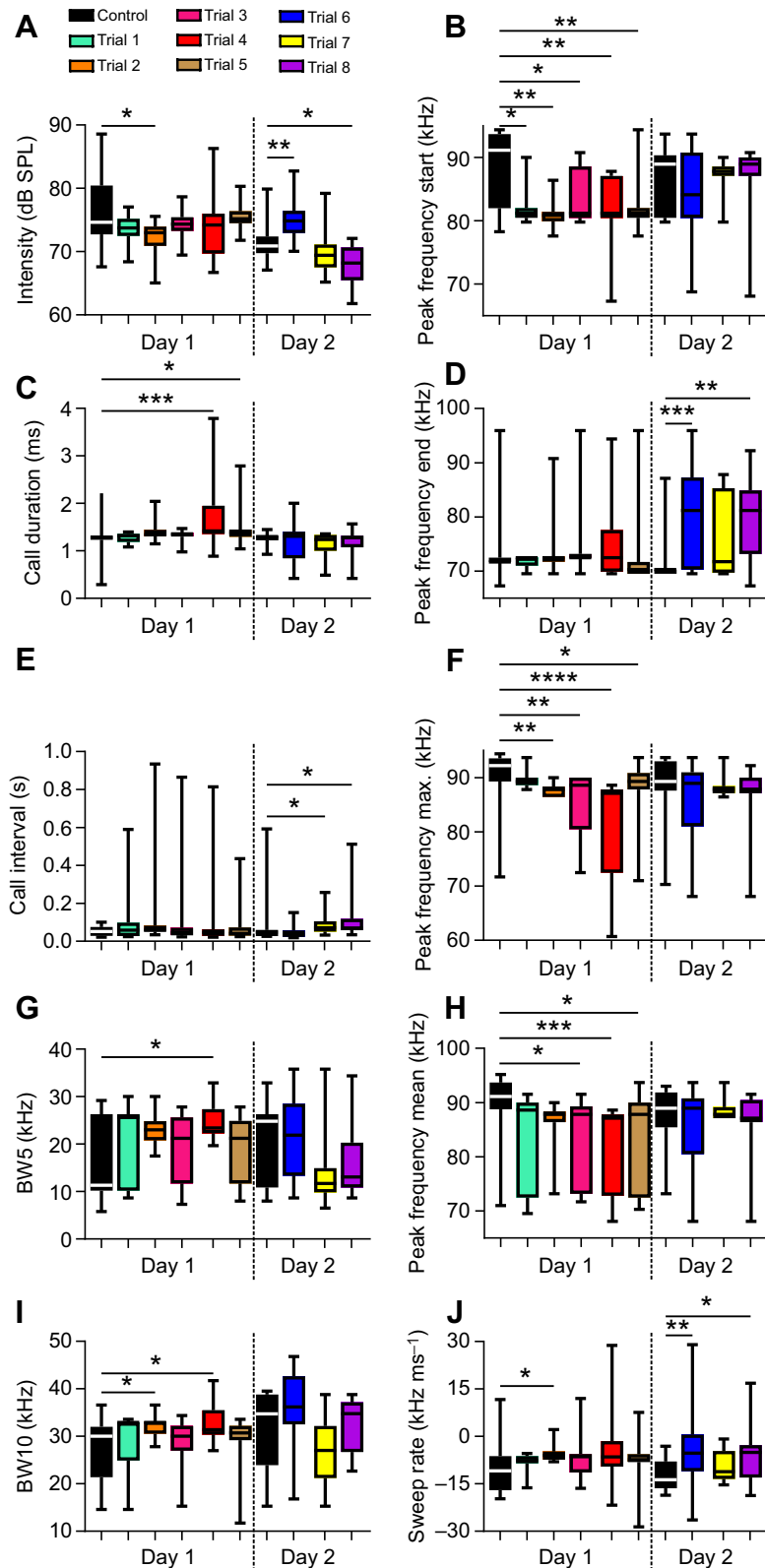
Fig. 7. Bats vary adjusted call parameters across trials and days. (A–J) Call parameters are shown as boxplots (whiskers represent minima and maxima, bars are medians and boxes show interquartile range) from each trial (8 test trials and 2 control trials) across two days (from one bat). For visualization purposes, each trial is color coded and the control trials are shown in black. Note that the bat changes some call parameters only at day 1 (e.g. peak frequency start; call duration; peak frequency max.) and not at day 2. Kruskal–Wallis Test and Dunn's multiple comparison *post hoc* test; *$P<0.05$; **$P<0.005$; ***$P<0.001$, ****$P<0.0001$. Number of calls: control day 1: $n=28$; trial 1=15; trial 2=10; trial 3=15; trial 4=17; trial 5=23; control day 2=18; trial 6=34; trial 7=16; trial 8=16.

all calls (889 calls from 69 trials and 10 animals), about three quarters of the calls (74.24%) showed abrupt adjustments in at least one call parameter (Fig. 8C). About half of the calls (50.84%) showed abrupt adjustments in more than one call parameter. The bats did not focus on a specific call parameter, but rather changed most of their parameters with equal probability (Fig. 8D). Call

intensity and call duration were least (7.24%) abruptly adjusted within the trials. To quantify if abrupt adjustments occur in response to the presence of the playback stimulus, we compared the call variability across subsequent calls during test trials with the call variability occurring during control trials (Fig. 8E). Thus, call variability normalized against the number of calls was
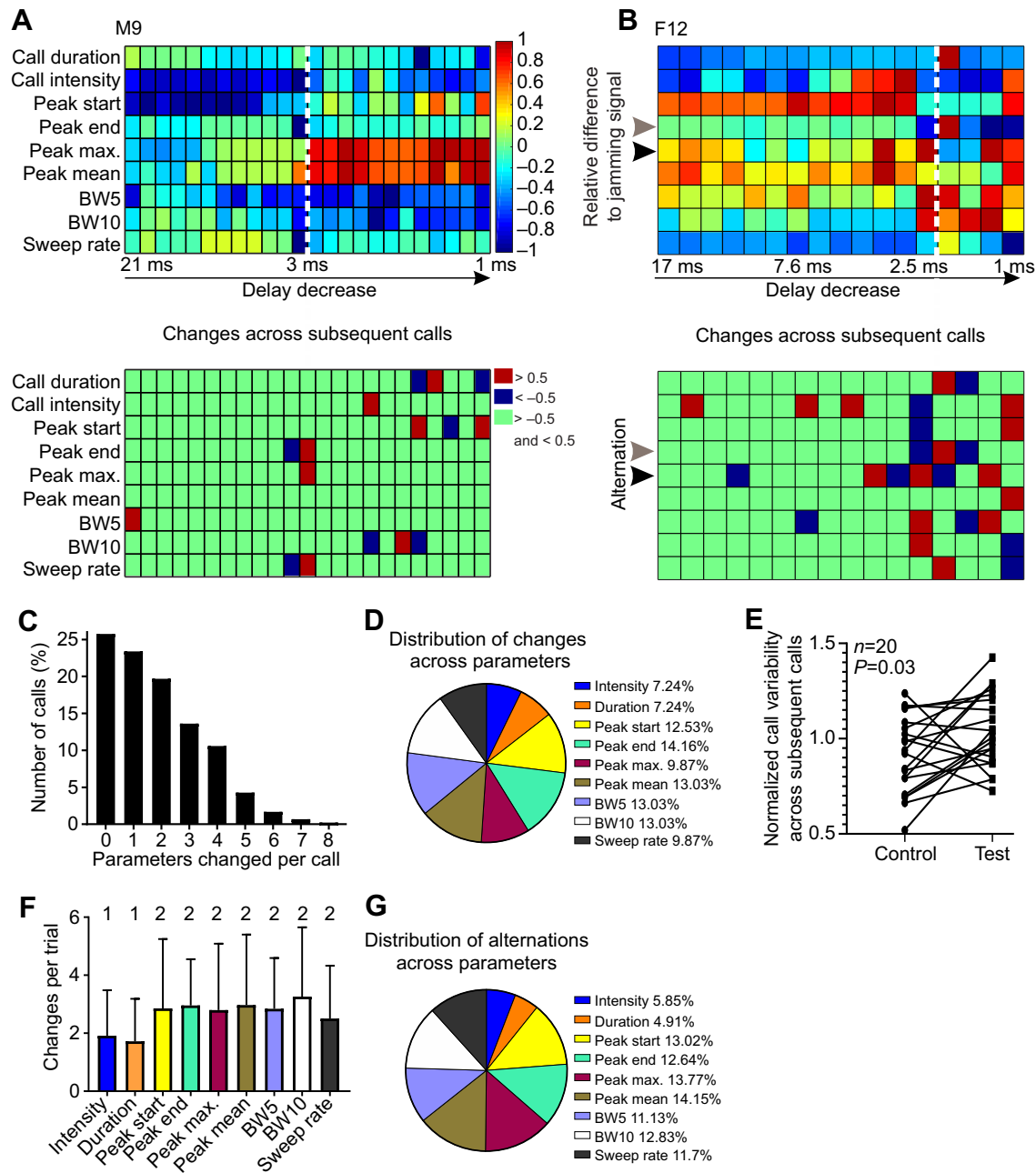
Fig. 8. Bats dynamically change echolocation parameters within trials. (A,B) Top color maps from two test trials (M9 in A and F12 in B), illustrating the differences between calls and playback in a call-wise manner. Along the *x*-axis, the calls are ordered according to the echo delay. Echo delays from some call-echo pairs are indicated in the *x*-axis. Along the *y*-axis, normalized call parameter differences are color coded. The differences were normalized to their absolute maximum at the corresponding parameter for the specific trial. In some trials, a clear transition of the adjusted call parameters can be detected (white vertical dashed lines). Occasionally, the bats alternate call values, as exemplified for F12 for the terminal and maximum peak frequency indicated by a gray and black arrowhead, respectively. Bottom color maps illustrate abrupt call adjustments across subsequent calls. Abrupt adjustments occurred when a call parameter between two consecutive calls varied by more than 50% (blue and red cells represent reductions or increases in the corresponding call parameter). Changes of the call parameters that are below 50% were not abrupt enough to be defined as a change (green cells). Transitions between strategies and alternations between call parameter values can be seen more easily in the lower color maps. (C) Histogram showing the level of parameters that are abruptly adjusted per call for all investigated calls (*n*=889). (D) Pie chart illustrating the distribution of abrupt adjustments over the call parameters. (E) Comparison of call variability across subsequent calls between control and test trials (*n*=20). Call variability across subsequent calls is higher for test than control trials (*P*=0.03; paired *t*-test) indicating that an increase of abrupt adjustments is associated with the presence of the playback. (F) Mean±s.d. number of changes per trial, plotted against the call parameter. Spectral parameters are shown to vary more often across trials than non-spectral ones (duration and intensity) (*P*<10^{-5} Kruskal–Wallis test; digits on top of each bar represents the statistical groups to which the bar belongs). (G) Pie chart representing the relative distribution of alternations across call parameters.

calculated for each day and each bat. Call variability was higher during test than in control trials (*P*=0.03; paired *t*-test).

When taking a closer look on the pattern of call changes over subsequent calls (color maps at the bottom of Fig. 8B), it became

clear that the bats sometimes changed the call parameters in an alternating manner. During the second half of the trial, the bat alternated between high and low terminal (peak end) and maximum peak frequencies (peak max.), indicated by gray and black

arrowheads, respectively. Before analyzing the alternations in more detail, we questioned how often the bats changed a certain call parameter during the trial. The bar plot in Fig. 8F shows that the bats changed spectral parameters more often per trial (peak start=2.85± 2.39; peak end=2.96±1.59; peak max.=2.8±2.29; BW5=2.84±1.75; BW10=3.26±2.39; sweep rate=2.51±1.82; all means±s.d.) than the call intensity (1.91±1.57) and the call duration (1.73±1.46; $P<10^{-5}$ Kruskal–Wallis test). Because spectral parameters varied more often during the trials, alternations occurred with a higher probability in spectral than in non-spectral (call intensity and call duration) parameters (Fig. 8G). Across the spectral parameters, the probability of alternations did not differ significantly ($P=0.91$ Kruskal–Wallis test), indicating that alternations could equally occur in each of the analyzed call parameters.

DISCUSSION

The present study describes echolocation adjustments that the frugivorous bat *C. perspicillata* shows in response to playback stimuli. Bats not only adjust echolocation parameters in response to acoustic interferers, but also when approaching obstacles or transiting between different locales (Neuweiler, 1990). Thus, we tested for the influence of acoustic interference under an otherwise invariant environmental context. The pendulum paradigm fulfilled these requirements because the behavioral scenario of an approach flight could be repeated (Fig. 2).

C. perspicillata decreased the terminal call frequency in response to the playback (Fig. 4). In addition, each bat adjusted different combinations of parameters (Table 1 and Fig. 6). To our surprise, the bats changed the adjusted parameters across trials (Table S4, Fig. 7) and even within trials (Fig. 8). This flexibility renders the echolocation behavior, in the presence of acoustic interferers, highly dynamic and unique across different individuals and time points. Utilizing such dynamics, the bats create unique echolocation streams that could facilitate signal extraction in noisy environments. This remains a hypothesis because the pendulum paradigm did not include a detection task with which we could have assessed the bat's echolocation performance. Alternatively, the pendulum paradigm triggers a calling variability in frugivorous bats that is different from that described in insectivorous bats (Kobler et al., 1985; Macias et al., 2016).

Acoustic interference for echolocating bats

For echolocating bats, the greater the similarity is between biosonar signals of conspecifics, the more challenging signal extraction becomes. To quantify the risk of acoustic interference that *C. perspicillata* may have to cope with, we compared the call designs from 32 individuals tested in the pendulum (Fig. 3). This comparison clearly shows that the echolocation calls across individuals are highly similar indicating that *C. perspicillata* may poorly rely on individualized call designs when navigating in the presence of conspecifics. These results stand in contrast to results obtained in the insectivorous bat *Myotis myotis* that could discriminate call designs from different individuals (Yovel et al., 2009).

For call-echo assignments, it has been proposed that bats keep an 'internal copy' of their broadcasted calls and compare that copy with received echoes (Simmons, 2012). Neural activity occurring before biosonar production in frontal and striatal brain regions could contribute to the formation of an internal copy (Weineck et al., 2020). Since echolocation calls across individuals of *C. perspicillata* are highly similar (this study), a correct call-echo assignment based on an internal copy is quite challenging in the presence of conspecifics. This has already been shown with neural data from the auditory cortex of *C. perspicillata* (Beetz et al., 2018).

In the cortex, distance processing gets degraded when an echolocation call of a conspecific reaches the bat's ears before the echo of its previously emitted call arrives. Therefore, the longer the echo delay, the higher the risk of being jammed.

Repertoire of echolocation adjustments in response to interfering signals and their possible neural correlates

To facilitate signal extraction, bats show many adaptations (Corcoran and Moss, 2017; Ulanovsky and Moss, 2008). These comprise motor responses, such as orienting sensory organs towards relevant signals (Eckmeier et al., 2008; Ganguly and Kleinfeld, 2004; Land, 2015; Ribak et al., 2009; Schroeder et al., 2010; Tarsitano and Andrew, 1999; Towal and Hartmann, 2006; Wohlgemuth et al., 2016a). In the present study, motor responses were limited by tightly positioning the bats on the platform of the pendulum mass. Moreover, in our experiment, motor responses could barely prevent escaping from acoustic interference as we placed the jamming source close to the animal's head.

Bats also adjust their echolocation call to decrease the spectral overlap between signal and background. This adaptation was originally described in electrolocating fish (Bullock et al., 1972; Watanabe and Takeda, 1963). When encountering animals whose signal frequencies overlap with the fish's own signal frequency, the animals shift the signal frequencies away from each other. This behavior has been named the jamming avoidance response (JAR) and it reduces the signal interference with signals coming from conspecifics. JAR has also been demonstrated in different insectivorous bats (Gillam and McCracken, 2007; Gillam et al., 2007; Habersetzer, 1981; Hage et al., 2013; Ibáñez et al., 2004; Miller and Degn, 1981; Ratcliffe et al., 2004; Takahashi et al., 2014; Tressler and Smotherman, 2009; Ulanovsky et al., 2004) and for the first time in a frugivorous bat in the present study. However, in contrast to weakly electric fish, which try to keep an individual specific frequency band throughout navigation, bats dynamically adjust their call spectra in various situations. Bats adjust their calls when approaching an obstacle or when transiting between different habitats (Barchi et al., 2013; Falk et al., 2014; Griffin, 1953; Hiryu et al., 2010; Kalko, 1995; Kalko and Schnitzler, 1989; Knowles et al., 2015; Kothari et al., 2014; Petrites et al., 2009; Roverud and Grinnell, 1985a; Schnitzler et al., 1987; Simmons et al., 1978; Surlykke and Moss, 2000; Wheeler et al., 2016). Since frequency adjustments occur frequently and under various conditions, an adaptation that purely depends on a JAR may not be sufficient to orient collision free in the presence of signal interferers, a hypothesis that gets support from simulations (Mazar and Yovel, 2020). This idea is further supported by the fact, that some insectivorous bats do not shift their frequency in response to acoustic interference (Götze et al., 2016; Ulanovsky et al., 2004) and that frequency adjustments occurring in the presence of conspecifics can be explained by distance-dependent spectral adjustments of the echolocation calls (Cvikel et al., 2015).

Bats may also reduce acoustic interference through adjusting their call bandwidth or call duration. Some studies reported that bats lengthen their calls when flying in noisy environments (Amichai et al., 2015; Simmons, 2017; Simmons et al., 1979, 1975; Tressler and Smotherman, 2009). In the pendulum, *C. perspicillata* varied call bandwidth and call duration in both directions in response to the playback. Decreasing call bandwidth or call duration may, respectively, reduce the spectral or temporal overlap between signal and background (Luo et al., 2015). Lengthening the calls increases the risk of temporal overlap, but it could still be useful if small echo portions are sufficient to gain spatial information. An

increase of the call bandwidth could also facilitate signal extraction because spectral regions that are not covered by the playback could specifically be occupied by the bat's signal. Bats also increase the signal-to-noise ratio by increasing call intensity (Amichai et al., 2015; Hage et al., 2013; Luo et al., 2015; Simmons, 2017; Simmons et al., 1978; Takahashi et al., 2014; Tressler and Smotherman, 2009; present study). Unexpectedly, in the present study, sometimes the bats decreased their call intensity in the presence of the playback. Although this decreases the signal-to-noise ratio, it could still be useful from the perspective of neuronal processing. Many auditory neurons are specialized to respond to faint biosonar signals while being insensitive to intense stimuli (Barone et al., 1996; Hechavarría and Kössl, 2014; Park and Pollak, 1993; Suga and Manabe, 1982; Yang et al., 1992).

In addition to adjustments to call design, but also changes in the emission pattern could reduce signal interference. Some bat species alternate between two call designs that differ in their frequency spectrum (Obrist, 1995; Roverud and Grinnell, 1985a,b). This adaptation allows a higher call rate by emitting a pair of calls before receiving an echo from the first call of the pair (Behr and von Helversen, 2004; Jung et al., 2007). The arising echoes differ in their frequency spectra which makes their discrimination feasible (Hiryu et al., 2010). Alternation of spectral call parameters have also been observed in the present study (Fig. 8B,F). However, these alternations occurred occasionally and not throughout the entire trial. Thus, the behavioral importance of alternating call parameters in *C. perspicillata* needs to be further assessed.

Some species reduce their call rate (Adams et al., 2017), or even cease to emit calls (Jarvis et al., 2013). This adjustment may be beneficial if the bats eavesdrop on echolocation signals from conspecifics and use the signals for orientation (Barclay, 1982; Chiu et al., 2008; Leonard and Fenton, 1984; Lin and Abaid, 2015). Although, *C. perspicillata* emitted fewer calls during test trials compared with control trials, the pendulum paradigm was not designed to test for eavesdropping on the playback stimulus.

Lastly, some bats increase their rate of grouping calls when orienting in cluttered or noisy environments (Beetz et al., 2018, 2019; Luo et al., 2015; Roverud and Grinnell, 1985a and present study). Grouping calls may improve echolocation performance in different ways. First, a defined periodicity of echo arrivals allows echo identification based on prediction (Petrites et al., 2009; Suga et al., 1983; Wheeler et al., 2016; Wohlgemuth et al., 2016a). Second, grouping the calls could create an information redundancy allowing the bats to rely only on a small portion of the call group (Beetz et al., 2018). Neurons of the bat's auditory midbrain and cortex can likely extract relevant echolocation information when the bats face such high call rates (Bartenstein et al., 2014; Beetz et al., 2018, 2016, 2017; Greiter and Firzlaff, 2017; Hechavarría et al., 2016; Macias et al., 2018; Sanderson and Simmons, 2005).

Bats show different combinations of adaptations when echolocating in the presence of an acoustic interferer

Instead of adjusting a single echolocation parameter when echolocating in noisy environments, our results indicate that *C. perspicillata* combines different adjustments. Combinatory adjustments of different call parameters in response to noisy environment have been reported in other insectivorous (Hage et al., 2013) and frugivorous bats (Luo et al., 2015). These combinations were, as in our study, uncoupled, meaning that each parameter was independently adjusted. Recent simulations demonstrated that frequency, intensity and duration adjustments have no or mild (only for increasing call intensity) effects in reducing signal

interference in hunting insectivorous bats (Mazar and Yovel, 2020). To our knowledge, these simulations did not consider the possibility that adjustments could be combined to assist signal extraction in noisy environments.

The dynamics and variability of echolocation adjustments could explain the high diversity of behavioral adaptations reported in earlier studies. However, the adjustments shown by bats could still be species specific, and only some species may adjust different echolocation parameters in combination. Note that this study was conducted in a fruit-eating bat. Most previous research has focused on insect-eating bats (Corcoran and Moss, 2017; Jones and Conner, 2019). One should consider the possibility that not all bats respond in the same way to acoustic interference.

In conclusion, the possibility that bats dynamically combine different echolocation adjustments to ensure signal extraction in noisy environments should be considered in future studies. The results from the present study indicate that *C. perspicillata* flexibly adjust different call parameters in response to playback stimuli. Future behavioral studies need to be performed to test if these adjustments facilitate signal extraction in *C. perspicillata* and other fruit- and insect-eating bats.

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